VIRUSES

by

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by

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CHAPTER I

VIRUSES AS MOLECULES

Shortly after the middle of the last century, Louis Pasteur succeeded in demonstrating that diseases could be caused by specific pathogenic bacteria. This was a startling discovery, one which inspired scholars the world over to seek for bacteria as the causative agents of disease. During the thirty years that followed Pasteur's demonstration, a great many of the common maladies of man and other animals and even plants were shown definitely to be caused by specific microorganisms - living bodies which could be cultured artificially and which were large enough to be seen with a good microscope and to be retained by certain types of filters. In view of this background, it can be appreciated that the Russian, Iwanowski, must have been exceedingly puzzled when he discovered, in 1892, that if he passed the juice from tobacco plants diseased with tobacco mosaic through one of these filters which would hold back all of the living organisms known at that time, the infectious principle was not held back, but passed through with the fluids. This observation simply did not fit in with the spirit of the times, and Iwanowski ignored his finding. Six years later a Dutchman named Beijerinck, having confirmed this observation, realized that the infectious principle of tobacco mosaic differed from ordinary bacteria and described it as a "contagium vivum fluidum". Thus, the vast field of filterable virus research was opened just before the beginning of the present century through the study of tobacco mosaic - a disease destined to become of unusual scientific interest.

Since tobacco mosaic virus will be the subject of considerable subsequent discussion, it might be well to examine the symptoms of a tobacco plant diseased with tobacco mosaic. A healthy tobacco plant has dark green, symmetrical, smooth leaves. A diseased plant is stunted and has leaves which are unsymmetrical, mottled and distorted. Figure 1 is a picture of a leaf taken from a tobacco plant diseased with tobacco mosaic.

FIGURE 1 - TOBACCO LEAF SHOWING SYMPTOMS OF TOBACCO MOSAIC. BRIGHT YELLOW SPOT IS RESULT OF MUTATION. (L.O.Kunkel, Ann. Rep. Quebec Soc. Proc. Plants 23, 25 (1934)).

One of the most outstanding properties of the causative virus is its extreme stability. Tobacco mosaic virus is so stable that it is able to survive the processing given commercial tobacco. It can be obtained fairly regularly by merely rubbing the leaf of a healthy tobacco plant with a moistened cigarette.

Beijerinck's revelation, that disease could be caused by agents different from ordinary bacteria, was almost as shocking as Pasteur's earlier discovery. It inspired a search for other viruses as the possible causative agent of other diseases of plants and animals. The result was that many virus diseases were discovered, and many of the properties of the filterable agents responsible were determined. Included among these virus diseases are small pox, sleeping sickness, yellow fever, infantile paralysis, measles, mumps, dengue, influenza, and fever blisters in man, foot and mouth disease and pox of cattle, rabies and distemper of dogs, horse encephalitis, hog cholera, pox and various tumors of fowl, many mosaic and yellow diseases of plants, and even the condition responsible for the unusual beauty of some tulips - a disease called tulip break. The agents responsible are usually fairly specific, for they cause disease only in a few hosts and are harmless in all others. Within the cells of the specific host, these viruses are multiplied, and occasionally

mutants are formed, giving rise to new viruses which cause slightly different diseases. The bright spot on the leaf pictured in Figure 1 is the result of a mutant having arisen at that point from tobacco mosaic virus. From this spot a new virus, one which produces a disease characterized by the development of bright yellow mottling, was isolated.

Before proceeding further, it would be desirable to formulate a definition of viruses. Viruses seem to be disease-causing agents, resembling somewhat ordinary bacteria, but differing from most bacteria in at lease two important respects; in their absolute dependence upon the living cells of the host for reproduction, and in their small size, usually below, but in a few instances, just at the lower limit of visibility with the microscope. No virus has ever been recognized which is not pathogenic, at least under some conditions. This may, of course, be attributed largely to the absence of criteria for their recognition other than pathogencity. Because they were too small to be seen, the true nature of these agents long remained a secret. It was only as recently as 1935 that any substantial clue concerning the real nature of these filterable, submicroscopic bodies was obtained, for in that year Stanley succeeded in isolating by chemical means from tobacco plants infected with this same tobacco mosaic a crystalline protein of high molecular weight possessing virus activity.

We know that the virus material is a protein because, like other proteins, such as egg albumin, it reacts with certain common specific reagents to give the usual color reactions expected of proteins. It can be precipitated by a whole series of reagents known to precipitate proteins. It can be denatured by heat and other means, i.e., it can be changed into a permanently insoluble form by a reaction analogous to that taking place when an egg is boiled. Finally, this material derived from mosaic diseased tobacco plants shows some of the characteristic immunological properties of proteins; that is, it can be used as an antigen and the resultant antibodies will undergo the precipitation reaction with the virus protein. All of these properties of the material leave no doubt that it is a protein. In addition to carbon, nitrogen, hydrogen and oxygen in the usual proportions, this virus protein is made up of 0.25% sulfur and 0.6% phosphorus. In common with all proteins, the building blocks are amino acids. Thus far, from fifteen to seventeen of the twenty or more common amino acids have been isolated or identified. They are alanine, arginine, aspartic acid, cysteine, glutamic acid, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, and valine, and possibly histidine and methionine. In addition to these amino acids, the virus also contains about 5% nucleic acid of the yeast or ribose type. The purines and pyrimidines of this nucleic acid have been isolated or identified.

This virus protein can be precipitated from solution by adjusting the acidity to the isoelectric point or by adding ammonium sulfate to a concentration of 20%. It separates in the form of needle-shaped paracrystals, visible with the microscope. A photo micrograph of crystals of tobacco mosaic virus protein is reproduced in Figure 2.

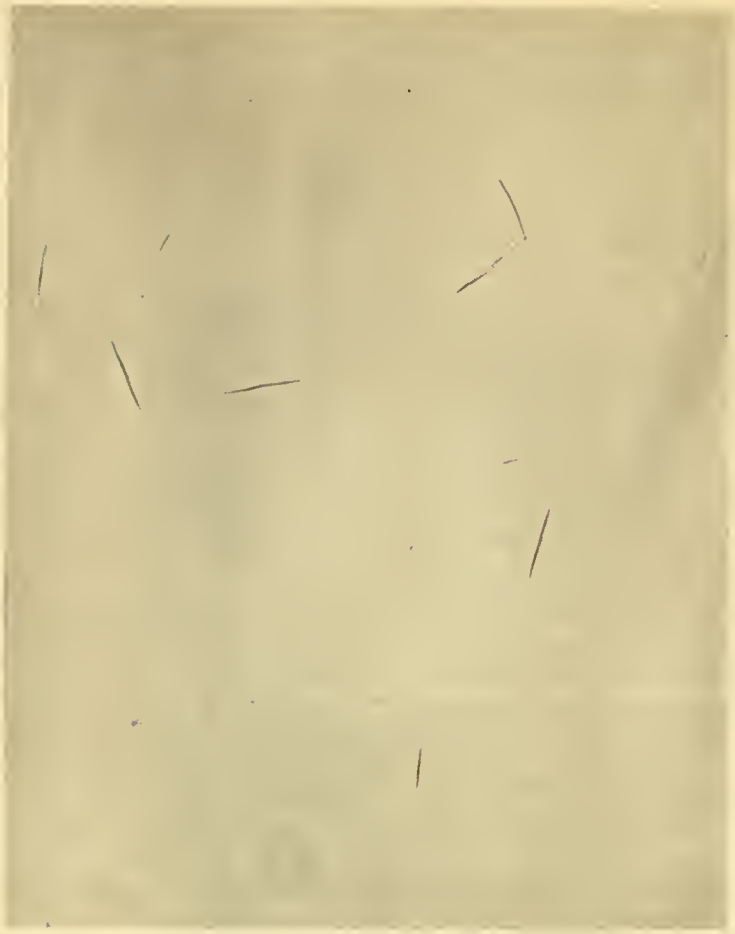

FIGURE 2 - PARACRYSTALS OF TOBACCO MOSAIC VIRUS PROTEIN. X675. (W. M. Stanley, Ergeb. Physiol. 39, 294 (1937)).

It must be emphasized that this material is in no sense an ordinary substance, for if these crystals are dissolved in water and then diluted a million fold, the solution will still be highly infectious and will institute disease in tobacco plants.

This picture gives a clue as to how purified tobacco mosaic virus can be obtained. Young tobacco plants are infected and allowed to grow for 3 to 8 weeks, and then they are harvested, frozen and ground. The juice is expressed the virus is precipitated out by the addition of ammonium sulfate. After re-precipitation a few times, the crystalline form is obtained. This is approximately the way Stanley first obtained purified tobacco mosaic virus, but the method is a bit too severe. Today there is a much better means of obtaining the material. Because of its high molecular weight, a value in the millions, it is possible to isolate this material from plant juice by simply centrifuging at high speed.

The yield of tobacco mosaic virus from diseased tobacco plants is amazingly high, about three parts per thousand of the plant material. Some of the virus is stored in the plant tissues in the form of both amorphous and crystalline inclusion bodies. A photomicrograph of hexagonal plate-like inclusion bodies, composed largely of stored virus, inside one of the hair cells of a mosaic diseased plant is reproduced as Figure 3.

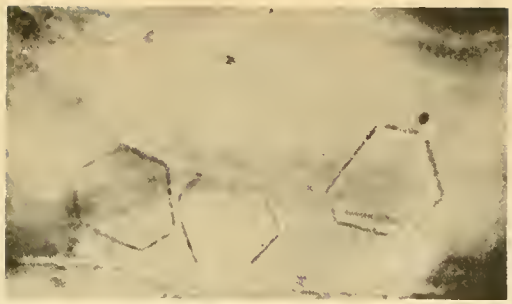

FIGURE 3 - HEXAGONAL PLATE CRYSTALS OF TOBACCO MOSAIC VIRUS IN EPIDERMAL CELL OF PETUNIA. (H.P. Beale, Contrib. Boyce Thompson Inst. 8, 413 (1937)).

It is interesting to observe that in the interior of the plant cell tobacco mosaic virus crystallize in the form of hexagonal plates but in the beaker in the form of long slender needles.

The first question to come to mind is whether this macromolecular nucleoprotein obtained from mosaic diseased tobacco plants actually is the virus or merely some substance contaminated by the virus. When a bacteriologist is confronted with the task of making a decision of this sort, he attempts to apply the criteria suggested by Koch. If the suspected organism is to be regarded as the etiologic agent of a given disease, it must always be associated with the disease; it must be isolated in pure culture on artificial media; the disease must be reproduced when a suitable host is inoculated with the pure culture; and finally the organism must be reisolated in pure form from the experimental host. The evidence amassed by Stanley to prove that the virus protein is the etiologic agent of tobacco mosaic virus can be organized to follow this generally accepted procedure fairly closely. The virus protein meets the first of Koch's criteria, for it has been isolated from many different batches of diseased tobacco plants and from other plant species infected with the virus. We know that it is the same protein in all of these cases, because the various preparations possessed roughly the same chemical, physical, biological and immunological properties. It can be accepted as a fact, then, that the virus protein is always associated with the tobacco mosaic disease. The second criterion can not be applied literally, for the viruses can not be cultivated on artificial media. However, the significance of the second criterion as applied to bacteria is derived from the fact that this procedure constitutes a demonstration that the organism under question is not contaminated by some other form, that is, that it is pure. It has been possible to apply other tests of purity and to demonstrate beyond reasonable doubt that tobacco mosaic virus protein can be obtained as an essentially pure chemical material. Some of the evidence in support of this conclusion may be summarized. The chemical and biological properties of the material remained unchanged following attempts to fractionate by various procedures; it is not possible to demonstrate the presence of an impurity by sensitive immunological reactions; and the material is essentially homogeneous with respect to various physical criteria such as sedimentation rate and electrophoretic behavior. Virus activity has never been obtained free from the protein. The ultraviolet absorption spectrum of the protein coincides with the destruction spectrum of virus activity, and the pH stability ranges of the protein and of virus activity coincide. This evidence of the purity of tobacco mosaic virus protein seems every bit as conclusive as that usually obtained by bacteriologists in applying Koch's second criterion. The last two criteria can be applied easily, for the virus protein is capable of causing the typical disease symptoms in plants, of being multiplied within them, and of reisolation from infected plants. Therefore, it seems reasonable to conclude, on the basis of this evidence, that tobacco mosaic virus nucleoprotein

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satisfies the requirements demanded by bacteriologists for acceptance as the causative agent of the mosaic disease.

The ultracentrifuge can be used to provide further evidence for the identity of the protein and the infectious principle of tobacco mosaic. A special type of ultracentrifuge cell designed by Tiselius, Pedersen, and Svedberg is used. Longitudinal and cross-sectional views are shown in Figure 4.

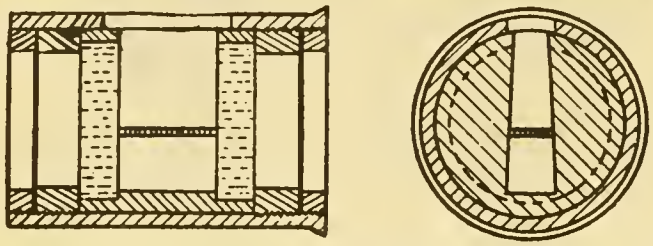


FIGURE 4 - LONGITUDINAL AND CROSS-SECTIONAL VIEWS OF SEPARATION CELL. (Svedberg and Pedersen "The Ultracentrifuge").

The cell consists of the usual plastic block with a sector-shaped slot and two quartz windows. It differs from the ordinary centrifuge cell by having a perforated barrier about 2/3 of the way from the top to the bottom. The barrier supports a piece of filter paper. After it is filled with virus solution, the cell is placed in the rotor. When the centrifuge is in operation, the particles sediment in the direction indicated as top-to-bottom on the diagram. The virus particles which come into the region of the barrier pass through it. A reasonably accurate sample of the material remaining above the barrier at the conclusion of the run can be removed for analysis by chemical or biological means. From such analysis one can compute the rate of sedimentation of the biological or chemical entity. In addition, this cell permits the material to be observed by the usual optical methods during the course of the run.

In an experiment with tobacco mosaic virus, the special centrifuge cell was filled with virus solution and was spun in the ultracentrifuge until the virus protein boundary was about 3/4 of the way from the top of the cell to the perforated barrier. Pictures were taken by the usual optical means during the experiment. After the completion of the run, the contents of the top section of the centrifuge cell were removed. From the optical data on the sedimenting boundary, the sedimentation constant of the nucleoprotein was calculated. Also from these data, the position of the boundary at the time the centrifuge stopped was determined. From the position of the boundary with respect to that of the barrier at the end of the experiment, it was a simple problem in solid geometry to calculate the fraction of the protein which moved out of the top compartment during the course of the sedimentation.

If the infectious principle sediments exactly as fast as the protein, the ratio of the infectivity of the contents of the top compartment to that of the original virus solution should be the same as the ratio of protein concentrations. Infectivity titrations were carried out both on the contents of the top compartment and on the original by the method of Price and Spencer. The results are presented in Table I.

TABLE I

FRACTION REMAINING ABOVE BARRIER AFTER CENTRIFUGATION

1. Protein determined optically	.26
2. Protein determined by chemical analysis	.26
3. Virus infectivity	.21 ± .045

It can be seen that the amount of protein remaining in the top compartment after the centrifuge was stopped was 26% of the original. It can also be seen that the virus infectivity remaining in the upper compartment was 21% of the original, with a probable error of 4.5%. This value, 21 ± 4.5% means that the chances are even that the correct value of the infectivity lies within the range 21 ± 4.5%. It is evident that the percentage of virus infectivity remaining above is the same as the percentage of protein remaining above, within the probable error of the determination.

In order to appreciate the full significance of this result, it is necessary to look at it from a slightly different point of view. It is possible to calculate the sedimentation constant of the infectious principle from the amount of infectivity remaining in the upper compartment. From the value of 21 ± 4.5% for the ratio of top compartment activity to original activity, one can calculate a sedimentation constant of $178 \pm 11 \times 10^{-13}$ for the infectious principle. This may be compared with the value, 165×10^{-13}, calculated from the optical data for the protein in this same experiment. The lower value is seen to be in excellent agreement with the sedimentation constant of the virus protein. This is really a fairly precise quantitative correlation of a biological function with a chemical entity. It is probably the most precise quantitative test for the identity of biological and chemical entities yet applied to tobacco mosaic virus. This result, taken in conjunction with the evidence previously presented, affords a very strong justification for making the assumption that the infectious principle of tobacco mosaic is in reality firmly associated with the nucleoprotein particles.

A second important question concerns the size and shape of such an unusual material as tobacco mosaic virus protein, the size and shape of the ultimate particle or molecule. It has already been shown that the virus protein particles are large enough to be sedimented rapidly in a high speed centrifuge. Therefore, the particles must be considerably larger than the molecules of ordinary proteins like egg albumin. But even before anyone knew how big the virus particles were, even before Stanley had isolated them and demonstrated their protein nature, Takahashi and Rawlins had figured out from stream double refraction studies that they were rod-like in shape. After the shape of tobacco mosaic virus was established, it became possible to compute, from the data of filtration, diffusion and centrifugation experiments and from other indirect physicochemical measurements, that tobacco mosaic virus particles are rod-like bodies about 12-15 mu thick and several hundred mu long, with a molecular weight of about 30 million. All of these computations were actually made before any one had ever seen a tobacco mosaic virus particle.

After the initial phases of these studies were completed, the electron microscope was perfected. This instrument uses electrons instead of light and electromagnets instead of lenses. Since a stream of electrons behaves like a beam of light with very short wave length, the lower limit of resolution with this instrument is far beyond that with ordinary light. An electron micrograph of tobacco mosaic virus particles is reproduced in Figure 5.

FIGURE 5 - ELECTRON MICROGRAPH OF TOBACCO MOSAIC VIRUS PARTICLES. (W. M. Stanley and T. F. Anderson, J. Biol. Chem. 139, 325 (1941)).

It can be seen that they actually are rod-like bodies with a thickness of 15 mu and a length of 270 mu, approximately the dimensions predicted from indirect studies.

Earlier it was mentioned that sometimes tobacco mosaic virus mutates to form new strains which cause new diseases. The resultant virus nucleoproteins are slightly different from that of ordinary tobacco mosaic virus. This can be illustrated by the case of a particularly interesting strain of tobacco mosaic virus which in nature causes a disease of ribgrass. This virus produces a disease in tobacco plants which, in its later stages, looks a bit like tobacco mosaic. Two stages are illustrated in Figure 6.

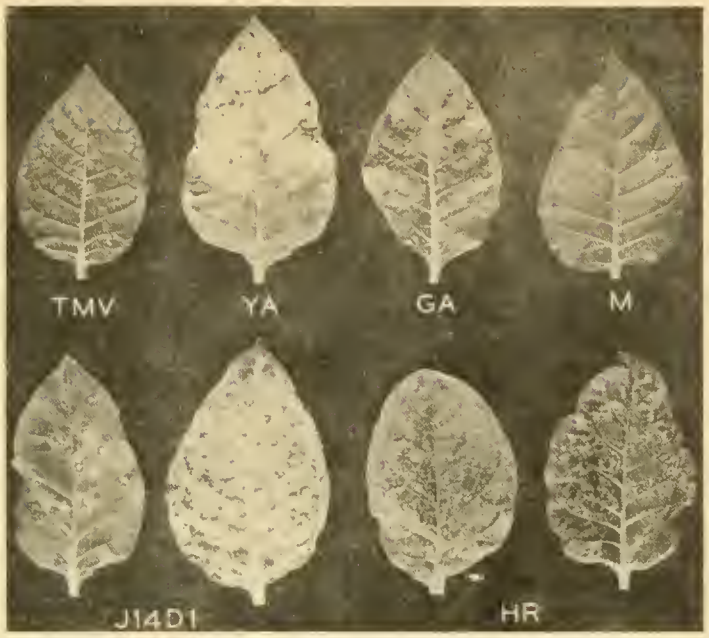


FIGURE 6 - LEAVES OF TOBACCO PLANTS DISEASED WITH VARIOUS STRAINS OF TOBACCO MOSAIC VIRUS. TMV-normal, YA-yellow aucuba, GA-green aucuba, M-Holmes'masked, J14D1- a derived mutant, and HR-ribgrass. (W. M. Stanley and C. A. Knight, Cold Spring Harbor Symposia on Quantitative Biology 9, 225 (1941)).

It differs from ordinary tobacco mosaic in that it produces a systemic infection in ribgrass, that is, an infection which spreads throughout the plant. It is believed that this virus is a strain of tobacco mosaic virus, because, if a tobacco plant is first infected with tobacco mosaic virus, it cannot be subsequently infected with ribgrass virus.

A nucleoprotein was isolated from tobacco plants infected with the ribgrass virus strain. When examined with the electron microscope, this virus was seen to be made up of rod-shaped particles very much like those of tobacco mosaic virus. However, when examined by other means, differences were observed. In the first place, the ribgrass virus was found to have a lower electrophoretic mobility than the tobacco mosaic virus. It is well known that if a protein is electrolyzed in a U tube, it will move towards the positive or the negative electrode with a characteristic speed for given conditions. Tobacco mosaic virus migrates toward the positive electrode faster than ribgrass virus under certain fixed conditions. This means that there must be some difference in the chemistry of the surface of the two viruses. However, even a more profound difference between the two virus proteins was demonstrated by Knight of the Rockefeller Institute. By careful chemical analysis he found that the amino acid composition of ribgrass virus differed from that of tobacco mosaic virus. The results are shown in Table II.

TABLE II

AROMATIC AMINO ACIDS AND PHOSPHORUS IN STRAINS OF TOBACCO MOSAIC VIRUS AND IN CUCUMBER VIRUSES 3 and 4

Virus	No. of preparations	Tyrosine	Tryptophane	Phenylalanine	P
		per cent	per cent	per cent	per cent
Tobacco mosaic.....	12	3.8	4.5	6.0	0.56
Yellow aucuba.....	3	3.9	4.2	6.3	0.52
Green aucuba	2	3.9	4.2	6.1	0.54
Holmes' ribgrass....	4	6.4	3.5	4.3	0.53
Holmes' masked.....	2	3.9	4.3	6.1	0.54
J14D1.....	2	3.8	4.4	6.1	0.55
Cucumber virus 4 ...	7	3.8	1.4	10.2	0.54
Cucumber virus 3 ...	1	4.0	1.5	10.0	0.56

It can be seen that the very building blocks of these two proteins are arranged in different proportions. This constitutes, then, a clear cut case in which slight but definite differencee in the biological activity of two strains of tobacco mosaic virus have their counterpart in slightly different chemical compositions of the virus proteins.

Thus far we have stayed pretty close to facts. Let us now permit our minds to wander a bit in order to picture the place of the viruses in our system of thinking. Are they matters vegetable, animal or mineral? A moment's reflection will lead to the realization that these materials stand in a unique position. On the one hand, they possess the properties of chemical compounds, materials much like ordinary egg albumin; and on the other hand, they are able to reproduce and even to mutate within the cells of living hosts and to cause injury to those hosts, simulating, in those respects, the behavior of pathogenic living organisms. They stand in the border zone between substances which are living and those which are non-living. Some people feel that viruses are simply chemical compounds, and they explain the ability to reproduce in the cells of a living host as being due to an auto-catalytic synthesis from raw materials present in the host cell, i.e., one virus particle introduced into a cell simply catalyzes the production of others like itself. Other theorists insist that the viruses are merely highly specialized living organisms whose material substances exhibit a few unusual chemical and physical properties. These two points of view are the extremes; many grades of opinion intermediate between them have been advanced. The truth of the matter is that there are no facts available which allow us to decide finally between the theories just advanced. Suffice it to say that, in a test tube, these materials behave not like living organisms but like chemical compounds. In a test tube, the viruses do not undergo respiration nor any other changes which cannot be accounted for in terms of simple chemical and physical laws.

Does the hypothesis that a virus is a protein molecule actually fit all of the facts? First, it is necessary to define the word molecule. The chemist defines a molecule as the smallest portion of matter that can exist in a free state, or as the smallest particle matter can be divided into without changing its chemical properties. Such a definition presupposes that all of the molecules of a given pure substance have identical chemical properties and are made up of the same number of atoms arranged in the same way. If, then, one is to prove that viruses are protein molecules, one must demonstrate that viruses are particles which cannot be subdivided without changing the chemical and bio-

chemical properties of the material and which are all of the same size and shape, a requirement necessitated by the stipulation that all molecules of a given substance be made up of the same number of atoms arranged in the same way.

It has already been seen from the electron microscope pictures that tobacco mosaic virus is particulate. The early work of Stanley, some of which was reviewed, showed most elegantly that these particles cannot be broken down into smaller units without destroying their most fundamental property,- infectivity. It is necessary only to demonstrate that these particles are all of the same size and shape in order to demonstrate that they can be molecules. If the electron micrograph of the virus is reconsidered, it can be seen that all of the particles are of the same shape, but alas, they are not quite all of the same size. Some are just a little longer than others. Hence we have failed on the very last step to demonstrate that tobacco mosaic virus particles are molecules in the strictest sense of the term. Some of us believe that some day it may be possible to obtain tobacco mosaic virus preparations which meet even this final criterion of homogensity. As a matter of fact, preparations which are very nearly homogeneous have recently been obtained. However, for the present it is necessary to consider tomato bushy stunt virus in order to demonstrate that viruses can be protein molecules.

Bushy stunt virus crystallizes in the form of beautiful dodecahedra, as illustrated in Figure 7.

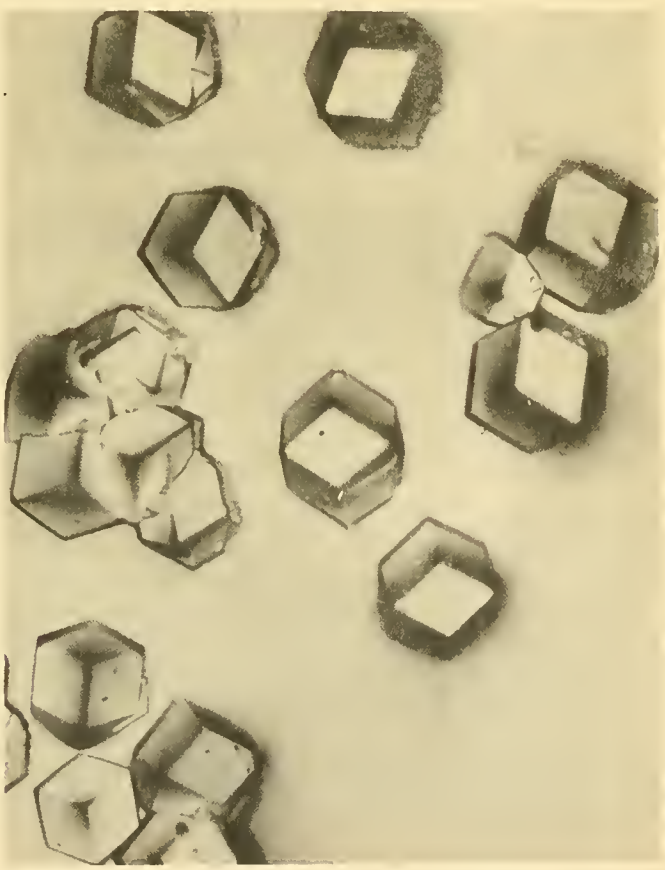


FIGURE 7 - CRYSTALS OF TOMATO BUSHY STUNT VIRUS.
(W.M.Stanley, J. Biol. Chem. 135, 437 (1940)).

The fact that it crystallizes in the form of dodecahedra, crystals belonging to the simplest class, the cubic, indicates that its particles are spherical, or nearly so. Recent studies with the electron microscope made by Price, Williams and Wyckoff show clearly that this deduction is correct, as illustrated by Figure 8.

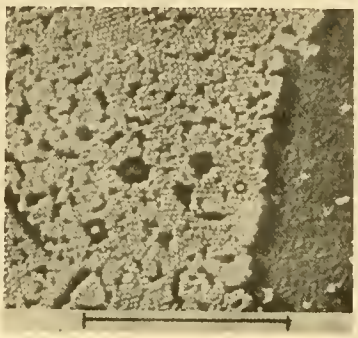


FIGURE 8 - SHADOWED ELECTRON MICROGRAPH OF TOMATO BUSHY STUNT VIRUS PARTICLES. (W. C. Price, R. C. Williams and R.W.G. Wyckoff, Arch. Biochem. 9, 175 (1946)).

The fact that these particles are spherical makes it relatively easy to test them for absolute homogeneity with respect to size and shape by means of the ultracentrifuge.

The rate at which a virus particle will sediment in an ultracentrifuge depends principally upon its size. The bigger the particle, the faster it will settle out. If, in a virus solution, all of the particles are of the same size, they will settle out at the same rate in an ultracentrifuge. This will result in the formation of a boundary between virus and liquid in the position of the uppermost layer of virus particles, a boundary which will move at exactly the rate of each and every particle. The most ideal type of boundary would be an infinitely sharp one;- such a one could be represented by a plane. In actual practice, however, boundaries are always more or less foggy. There are two possible reasons for this. First of all, the virus particles may not all be quite the same size. In such an event, the bigger particles in the boundary region will get ahead of the smaller ones and the boundary will naturally spread out. But, even if all of the particles are of exactly the same size, the boundary will still get foggy due to diffusion. Some of the particles in the solution region will diffuse up into the solvent region. Thus, the problem that confronts one in trying to decide by means of the ultracentrifuge whether or not a virus is absolutely homogeneous is obvious. The boundary spreading during the course of the sedimentation experiment must be measured by highly specialized optical methods, and then one must decide whether the observed boundary spreading is due to diffusion alone, or due to inhomogeneity of the particles. Just such studies were carried out with bushy stunt virus, and it was found that the observed boundary spreading could be accounted for quantitatively by the known diffusion rate of the particles. The same story can be told in the language of geometry, as illustrated in Figure 9.

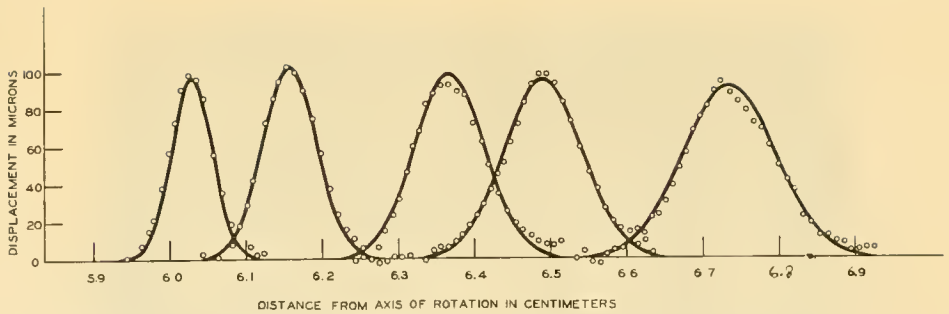


FIGURE 9 - BOUNDARY SPREADING IN SEDIMENTATION EXPERIMENT ON BUSHY STUNT VIRUS. OPEN CIRCLES ARE EXPERIMENTAL POINTS OBTAINED BY LAMM SCALE METHOD. SMOOTH CURVES ARE THEORETICAL BOUNDARIES CALCULATED FROM KNOWN DIFFUSION CONSTANT. (M.A.Lauffer, J. Biol. Chem. 149, 99 (1942)).

The curves seen there are simply a convenient way of describing quantitatively the position and the sharpness of a boundary in the ultracentrifuge after various periods of centrifugation. They tell us that after 85 minutes of centrifugation the virus - buffer boundary had moved to the first position, and after 220 minutes it had moved to the last position. The sharpness of these peaks tell us how sharp the boundary is at every stage. At first, it is fairly sharp, then it becomes more diffuse. The points represent the actual sharpness of the boundary in the centrifugation experiment. The curves represent the theoretical sharpness that one should get when the boundary spreading is due 100% to normal diffusion and not all to inhomogeneity. One can see perfectly well that the agreement between theoretical and actual boundary sharpness is very good indeed. This means, then, that the bushy stunt virus particles are absolutely homogeneous with respect to size and shape, or very nearly so.

Through the study of tobacco mosaic virus, it was possible to demonstrate that viruses are protein particles and that these particles are homogeneous chemically and identical in one dimension. However, it was not possible to obtain preparations in which the rod-like particles were all of exactly the same length. Hence, we failed on the very last step in our attempt to prove that tobacco mosaic virus particles are protein molecules. When bushy stunt virus protein was considered, it was possible to show with the ultracentrifuge that virus particles can all be of identical size and shape, thereby hurdling the last serious barrier to believing that viruses are indeed protein molecules.

Nevertheless, whether or not any viruses actually are molecules is a question which may never be settled. It was sufficient for the cause of science that the hypothesis that the viruses are molecules is not untenable, for this idea has been of the most tremendous significance. It has provided the inspiration for an entirely new approach to virus research. For forty years after the discovery of viruses, very little real progress was made in the elucidation of their nature. Almost all of the knowledge concerning the nature of viruses that we have today has been developed during the past ten years, since the concept of the molecular nature of viruses was given impetus by the crystallization of tobacco mosaic virus. Numerous viruses have since been obtained in crystaline form. These include many strains of tobacco mosaic virus, tomato bushy stunt virus, tobacco necrosis virus, southern bean mosaic virus, and turnip yellow mosaic virus. A photomicrograph of crystals of the bean mosaic virus and an electron micrograph of the particles of this virus are shown in Figures 10 and 11.

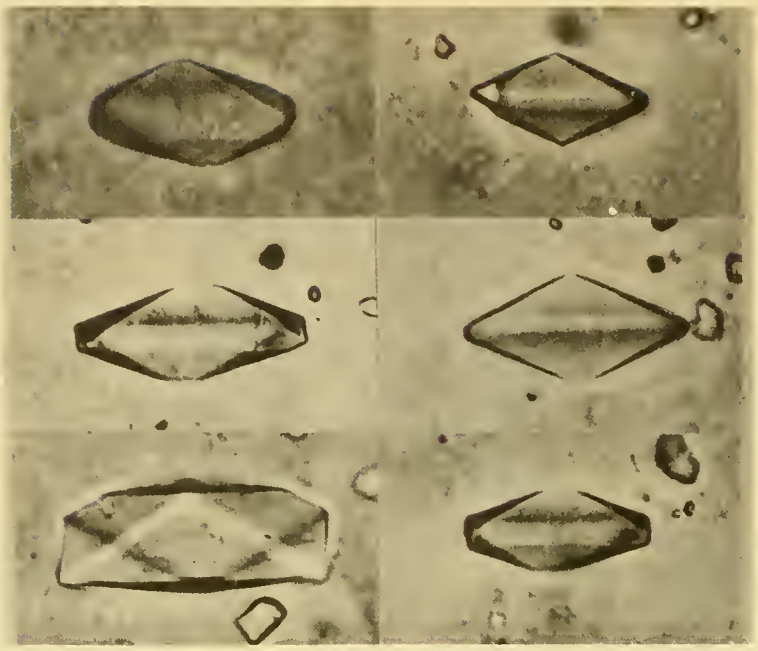


FIGURE 10 - CRYSTALS OF SOUTHERN BEAN VIRUS. (W.C.Price, Am. J. Bot. 43, 45 (1946)).

FIGURE 11 - SHADOWED ELECTRON MICROGRAPH OF PARTICLES OF SOUTHERN BEAN MOSAIC VIRUS. (W.C.Price, R.C.Williams and R.W.G.Wyckoff, Arch. Biochem. 9, 175 (1946)).

Several animal and human disease viruses have also been obtained in reasonably pure form. These include vaccinia virus, illustrated in Figure 12; influenza virus, illustrated in Figure 13; equine encephalitis virus, rabbit papilloma virus, illustrated in Figure 14; and several strains of poliomyelitis virus.

FIGURE 12 - SHADOWED ELECTRON MICROGRAPH OF VACCINIA VIRUS. (D.G.Sharp et.al., Proc. Soc. Exp. Biol. & Med. 61, 259 (1946)).

FIGURE 13 - SHADOWED ELECTRON MICROGRAPH OF INFLUENZA VIRUS. (R.C.Williams and R.W.G. Wyckoff, Proc. Soc. Exp. Biol. & Med. 58, 265 (1945)).

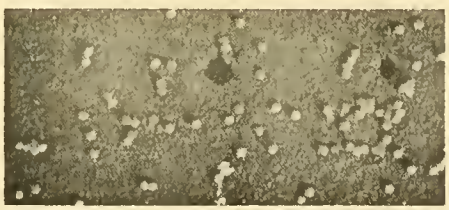


FIGURE 14 - SHADOWED ELECTRON MICROGRAPH OF RABBIT PAPPILOMA VIRUS. (D.G.Sharp et.al. Proc. Soc. Exp. Biol. & Med. 61, 259 (1945)).

Bacteriophage or bacterial viruses have also been isolated in pure form. An electron micrograph is shown in Figure 15.

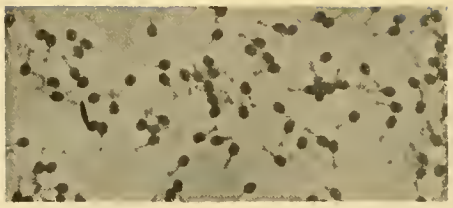


FIGURE 15 - ELECTRON MICROGRAPH OF THE T^2 BACTERIA OF E. COLI. (D.G.Sharp et.al., Proc. Soc. Exp. Biol. & Med. 61, 259 (1946)).

These purified preparations of viruses are the only sensible starting materials for many types of experiments designed to discover the nature of viruses. All of them were obtained by the application of physical and chemical procedures. The development of these procedures was an outgrowth of the concept that viruses are molecules. Thus, this concept has served the cause of virology magnificently. What matters it whether the concept is right or wrong?

CHAPTER II

THE SIZE AND SHAPE OF VIRUSES

In "Viruses as Molecules" it was seen that purified virus preparations can be obtained by chemical and physical means. Such preparations can be used to determine something of the nature of the virus particle. One of the first things we all want to know about a virus is its size and shape. Numerous physical methods are available for providing such information. They include ultrafiltration, ultracentrifugation, diffusion, viscosity, stream double refraction, x-ray diffraction, and electron microscopy. It would be impossible in the limited space available to examine the evidence on the size and shape of all of the viruses that have been investigated thus far. Rather than attempt this, it would be preferable to consider four viruses in more or less detail. The four chosen are tomato bushy stunt, southern bean mosaic, tobacco mosaic and influenza viruses.

Tomato Bushy Stunt Virus

Let us see first how the tools of the physical chemist can be applied to the study of tomato bushy stunt virus. The most spectacular tool for the investigation of viruses is the electron microscope. As was mentioned previously, the electron microscope is a device which resembles the optical microscope in principle, but which uses a stream of electrons instead of a beam of light and magnetic or electric fields instead of lenses. Because of the short effective wave length of the stream of electrons, the lower limit of resolution with the electron microscope is far below that with the ordinary microscope. Electron images are shadows. Matter scatters electrons. Those electrons scattered by an object do not reach the photographic plate. Therefore, a shadow image of the object is formed. The efficiency of matter in the scattering of electrons depends upon its density. Viruses have low density and therefore a low scattering power. For that reason their images have poor contrast. A technique has been developed to improve the contrast. A very thin film of a heavy metal, such as gold, is evaporated onto the virus particles at an angle. Then the particles are micrographed. The effect is to produce images of particles with shadows comparable to the effects produced by photographing landscapes in oblique illumination. Figure 8 is an electron micrograph of bushy stunt virus obtained by the shadow technique. It can be seen that the particles are approximately spherical bodies. The size has been determined as about 25 millimicrons.

More detailed information can be obtained by other physical methods. Among these, the most important is the ultracentrifuge. Let us recall momentarily what happens to a virus particle in a centrifugation experiment. When a particle moves through a viscous medium in a centrifugal field, it is subjected to two forces - a centrifugal force, F_c, tending to increase its speed, and a frictional force, F_r, tending to decrease its speed. The particle moves at a uniform rate when these two forces exactly balance each other. Now, the first of these is a function of the magnitude, g, of the centrifugal field and the effective mass, $(m_p - m_s)$, of the particle, whereas the second is a function of speed, v, with which the particle moves, and the coefficient of friction, f, of the particle. Since, according to Stoke's law, this friction coefficient is directly proportional to the radius of a spherical particle, it is relatively simple to calculate the size of a spherical particle from its sedimentation rate in a known centrifugal field. These ideas can be summarized:

$$F_c = g(m_p - m_s) \quad (1)$$

$$F_r = vf \quad (2)$$

$F_c = F_r$ when the particle moves along at a uniform rate.

Therefore, $$vf = g(m_p - m_s) \quad (3)$$

Hence, $$v = \frac{g(m_p - m_s)}{f} = \frac{\omega^2 x(m_p - m_s)}{f} \quad (4)$$

The symbols ω and x represent the angular velocity of the centrifuge and the distance of the particle from the axis of rotation, respectively. This equation can be simplified if the particles are unhydrated spheres, for Stoke's law states that $f = 6\pi r\eta$, where r is the radius and η is the coefficient of viscosity. Also, for a sphere $m_p = 4/3\pi r^3 d_p$ and $m_s = 4/3\pi r^3 d_s$, where m_p and m_s represent the masses, and d_p and d_s the densities of the particles and the displaced solvent, respectively.

Therefore, for a spherical particle with radius r,

$$v = \frac{\omega^2 x\ (4/3\pi r^3 d_p - 4/3\pi r^3 d_s)}{6\pi r\eta} \quad \text{or}$$

$$v = \frac{2/9\ \omega^2 x\ r^2 (d_p - d_s)}{\eta} \quad (5)$$

Most investigators reporting sedimentation studies talk about sedimentation constants. This latter is defined as the sedimentation rate in unit centrifugal field and is designated as

$$S = \frac{v}{\omega^2} = \frac{2r^2(d_p - d_s)}{9\eta} \quad (6)$$

In a centrifugation experiment, if all of the particles move at about the same rate, a boundary between solvent and solution is formed, which moves at the rate of each particle. As was seen previously, the ideal boundary would be an infinitely sharp one, but in practice more or less fuzzy ones are always obtained. Figure 9 shows graphically the sedimenting boundary at various times in an experiment on bushy stunt virus. The position of the center of the curves tells us how foggy it is. From the rate of displacement of this boundary in a known centrifugal field, the sedimentation constant of bushy stunt virus can be calculated. It is, on the average, 132×10^{-13} cm./sec. in unit field.

If it is assumed that the particles are unhydrated spheres, one can compute their size directly by substituting this sedimentation constant in equation (6). The only additional information needed is the viscosity of the solvent and the densities of the solvent and the particles. The first two quantities can be measured directly and the third can be computed from the density of a solution of known composition. Ordinarily one speaks in terms of the partial specific volume of a virus particle. This is essentially the reciprocal of the anhydrous density. McFarlane and Kekwick determined the partial specific volume of the particles to be 0.74 . This corresponds to a density of 1.36 . From these data the radius can be obtained. Then, from the radius, it is a simple matter to compute the molecular weight. Calculated in this manner, the molecular weight is 7.4×10^6 . The size of a spherical particle can also be obtained from diffusion data. According to the Einstein-Sutherland equation,

$$D = \frac{RT}{Nf} \qquad (7)$$

D is the diffusion constant, R, the gas constant, N, Avagadro's number, and T the absolute temperature. For the unhydrated sphere, according to Stoke's law, $f = 6\pi r\eta$; therefore,

$$D = \frac{RT}{6\pi\eta Nr} \qquad (8)$$

Thus the radius of an unhydrated sphere can be calculated from the diffusion constant. Then the molecular weight can be determined from the value for the radius and the density. Diffusion studies on bushy stunt virus were carried out by Neurath and Cooper. A value for D of 1.15×10^{-7} cm.^{2}/sec. was obtained. When this value is considered in conjunction with the value of 1.36 for the dry density of the virus, one obtains about 19×10^6 for the molecular weight. This does not agree at all with the value 7.4×10^6, calculated from sedimentation data. Obviously, something is wrong with the assumption that the bushy stunt virus particle is an unhydrated sphere. However, it is not necessary to make assumptions concerning the shape and the state of hydration of the virus particles in order to determine the molecular weight. This is true because the same friction factor which determines the rate of sedimentation of a virus also determines the rate of diffusion. Hence, by combining equations (4) and (7), the friction factor, f, can be eliminated entirely, and the particle weight can be expressed as a function of the sedimentation and the diffusion constant.

$$\frac{RT}{D} = \frac{\omega^2 x}{v} N = \frac{N}{s}(m_p m_s) \qquad (9)$$

Since $m_s = m_p \frac{d_s}{d_p}$ and $Nm_p = M$, where M is the molecular weight,

$$\frac{RT}{D} = \frac{1}{s} M\left(1 - \frac{d_s}{d_p}\right), \quad \text{or} \quad M = \frac{RTs}{D\left(1 - \frac{d_s}{d_p}\right)} \qquad (10)$$

In the diffusion constant, 1.15×10^{-7}, is used in conjunction with the sedimentation constant, 132×10^{-13}, in equation (10), a value for the molecular weight of 10.6×10^6 is obtained. The error has been estimated as $\pm$ 1,000,000. This is the true value of the anhydrous molecular weight. In order to explain the discrepancy between the value calculated from diffusion data and that calculated from sedimentation data, one would have to assume either that the particles are not spherical, or that they are hydrated spheres which have soaked up 77% of their own weight of water. Since we know that the particles are essentially spherical, we are forced to the conclusion that they are hydrated spheres.

X-ray diffraction studies have been made on crystals of bushy stunt virus by Bernal, Fankuchen and Riley. Because the crystals were too small to be examined singly, these workers studied powder diagrams obtained from suspensions of the crystals in their mother liquor. From these studies, they decided that the crystal structure consists of a face-centered cubic lattice, that there are two virus molecules per unit cell, and that the wet molecular weight is 22 million. Wet crystals shrink about 20% when dried in a vacuum. This shrinkage was verified by the decrease in the interplanar distances within the crystals, as measured by X-ray technique. From these considerations alone, it can be computed that in the wet crystals each gram of virus is associated with.67 grams

of water. Taking this into account, the wet molecular weight of 22 million would correspond to a dry molecular weight of about 12 million. This value is in reasonably good agreement with that obtained from diffusion and sedimentation, 10.6 ± 1 million. Also, the amount of water computed from X-ray data agrees with that obtained from sedimentation and diffusion data. Very recently, Oster used the method of light scattering to determine the molecular weight of bushy stunt virus. The principle of this method is that the amount of light scattered by a colloidal solution of spherical particles is proportional to the size of the particle. Oster obtained a value of 9 million for the anhydrous molecular weight of bushy stunt virus. Thus there are three estimates of the molecular weight of bushy stunt virus, 9 million from light scattering, 10.6 ± 1 million from sedimentation and diffusion, and 12 million from X-ray studies. The average would be 10.5 million. Further, the electron microscope shows that the particles are spheres. X-ray data indicate that the spheres in the crystals are 67% hydrated and sedimentation and diffusion data indicate that they are 77% hydrated. From the average molecular weight, 10.5 million, and the dry density, one can calculate that the diameter of the dry virus particle should be 29 millimicrons. Price, Williams and Wyckoff made numerous measurements of the diameter of the bushy stunt virus particles on electron micrographs. They obtained values ranging from 25 to 27 millimicrons. Thus we can see how the physical picture of the bushy stunt virus was built up. We know that it is a sperical particle with a dry molecular weight between 9 and 12 million and a dry diameter between 25 and 29 millimicrons, but that in solution, it contains between 67 and 77% water of hydration.

Pictures of the crystals of southern bean mosaic virus, recently crystallized by Price, and a gold shadow micrograph are shown in Figures 10 and 11. It is evident that the southern bean mosaic virus is also essentially spherical. Miller and Price have measured several of its physical constants. The sedimentation constant is $_{10}$-13 cm. per sec. per unit field, the diffusion constant is 1.34×10^{-7} $cm.^2/sec.$ and the partial specific volume is 0.70. From these data, again as in the manner employed in the case of bushy stunt virus, one can calculate that the southern bean mosaic virus is a hydrated sphere with 80% water of hydration, with a dry molecular weight of 6,630,000, and a dry radius of 26 mu.

Tobacco Mosaic Virus

We come now to a consideration of tobacco mosaic virus. A study with this virus was carried out at the Rockefeller Institute Laboratory with a single preparation. It was photographed with the electron microscope and shown to consist of rod-like particles with an average length of 270 millimicrons, as shown in Figure 5. The thickness of the virus particles as obtained from the electron micrograph is about 15 millimicrons. However, a more precise figure was obtained from X-ray diffraction studies carried out by Bernal and Fankuchen. These investigators found that the virus rods are lined up parallel to each other with hexagonal symmetry with respect to cross section, as illustrated in Figure 16.

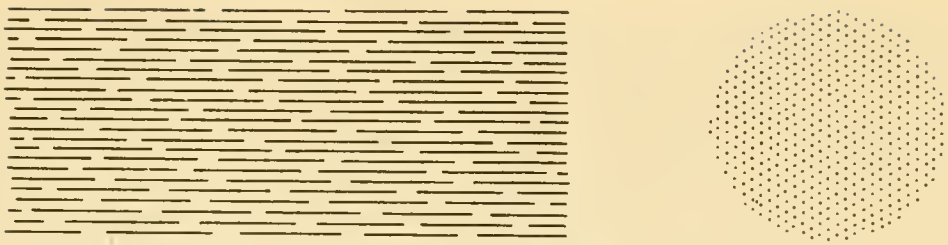


FIGURE 16 - THE ARRANGEMENT OF ROD-SHAPED PARTICLES OF TOBACCO MOSAIC VIRUS IN NEEDLE-SHAPED PARACRYSTALS, ACCORDING TO VIEWS OF BERNAL AND FANKUCHEN. LEFT, LONGITUDINAL DIAGRAM; RIGHT, CROSS SECTIONAL DIAGRAM. (M.A.Lauffer and W.M.Stanley, Chem. Rev. 24, 303 (1939)).

The minimum lateral spacing obtained with thoroughly dried virus crystals was found to be 15 millimicrons. This value affords a precise estimate of the maximum diameter that the virus rods possess in the dried state. Thus, from a combination of electron microscope and X-ray diffraction data, it is known that the particles of the particular tobacco mosaic virus preparation under study have an average length of 270 millimicrons and an average diameter of 15 millimicrons. Since these dimensions were obtained by straight-forward methods, they can be accepted as direct measures of the size and shape of the virus particles.

This same preparation of the tobacco mosaic virus was also subjected to diffusion, sedimentation, viscosity and partial specific volume studies. It is a well-established fact that the molecular weight of a particle, regardless of its shape, can be determined by the method of Svedberg, employing equation (10), from the diffusion constant, the sedimentation constant and the partial specific volume. From the data obtained, a value for the molecular weight of tobacco mosaic virus of 31.6 million was computed. In view of present day concepts, it is possible to interpret these data not only in terms of the molecular weight, but also in terms of the shape of the virus particles. The procedure is somewhat as follows. From the molecular weight, which has already been determined, and the partial specific volume, it is a simple matter to compute the molecular volume, and from it, the radius that the virus particle would have if it were an unhydrated sphere. From this, by Stoke's law, the friction coefficient the particle would have if it were an unhydrated sphere can be computed. In this calculation, no unwarranted assumption is made. Thus, one can regard the value of the friction coefficient of the hypothetical unhydrated sphere as an experimentally determined value. By using the Einstein-Sutherland equation, which has also been subjected to experimental verification in numerous cases, it is possible to determine directly the true friction coefficient of the particle. In the case of the tobacco mosaic virus under consideration, the actual coefficient of friction turned out to be 7.6×10^{-7} grams per second, and that which the particle would have were it an unhydrated sphere turned out to be 3.8×10^{7} grams per second. When the former is divided by the latter, one obtains a value of 2.0 for the friction ratio of the particle. From an extension of the theoretical considerations used by Stokes for calculating the coefficient of friction of a spherical particle, Gans, Herzog and finally Perrin contributed to the development of equations which relate the friction ratio to the shape of the particles. These investigators assumed that rod-like or plate-like particles have shapes which can be represented by elongated or flattened ellipsoids of revolution. The equations obtained show the relation between the friction ratio and the ratio of the long to the short semi-axes of the ellipsoids of revolution. Entirely different equations are obtained for rod-like and plate-like particles. However, if independent evidence is available to guide one in his choice of a rod-like or plate-like model, one can calculate

the ratio of the length of the long semi-axis to that of the short semi-axis from the friction ratio. For a rod-like ellipsoid of revolution, this is approximately the equivalent of the ratio of length to diameter. When the friction ratio of tobacco mosaic virus, 2.0, is substituted into the equation obtained for rod-like particles, one obtains a value of 18.6 for the ratio of length to thickness. The molecular volume of the tobacco mosaic virus particle is already known. Therefore, since we know the ratio of length to thickness and the volume, it is a simple problem in solid geometry to calculate the actual dimensions of the virus particle. Values of 256 millimicrons for the length and 13.8 millimicrons for the thickness are obtained.

According to recent theories also developed from hydrodynamic considerations, it is possible to determine the shape of suspended particles from measurements of the intrinsic viscosities of solutions of those particles. The intrinsic viscosity is a measure of the increase in relative viscosity imparted to a solution by dissolving unit volume of a solute. Several such theoretical developments are summarized in Figure 17.

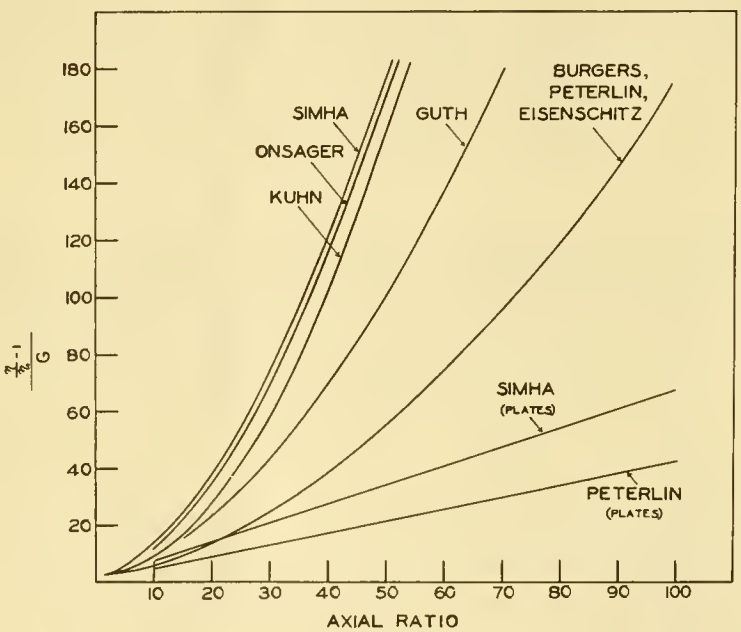


FIGURE 17 - GRAPHICAL REPRESENTATION OF SEVERAL THEORETICAL RELATIONSHIPS BETWEEN INTRINSIC VISCOSITY AND THE SHAPE OF SUSPENDED PARTICLES. (M.A.Lauffer, Chem. Rev. 31, 561 (1942)).

It seems, at present, that the most adequate treatment is that of Onsager and of Simha. The intrinsic viscosity of the tobacco mosaic virus solution was found to be 39. If it is assumed that this virus particle is unhydrated and that the treatment of Onsager and of Simha is adequate, one can calculate a ratio of length to thickness for the tobacco mosaic virus particle of 20 to 1. It is possible to pursue this result obtained from viscosity measurements even further. Since one can compute the ratio of length to thickness of a particle from the friction ratio by means of the Gans-Herzog-Perrin equation, one can also do the reverse, that is, calculate the friction ratio from the ratio of length to thickness of a particle. Then, when the friction ratio and the partial specific volume are known, it is possible to determine the molecular weight from either diffusion or sedimentation measurements taken singly. From

diffusion measurements, and the Einstein-Sutherland equation (7), one obtains the true friction coefficient of a particle, and with this value and the value for the friction ratio, one can compute the friction ratio the particle would have if it were an unhydrated sphere. Then, by using Stoke's law, one can get the radius this particle would have if it were a sphere and from that and the partial specific volume, the molecular weight. Knowing the molecular weight in addition to the shape as determined by viscosity, one can calculate the actual dimension of the particle. This computation gives us the molecular weight and the particle dimension from diffusion and viscosity data taken in conjunction with the partial specific volume. With known values for the friction ratio and the partial specific volume, one can also determine the molecular weight and particle dimensions from sedimentation and partial specific volume data. The sedimentation rate depends upon the particle mass and upon the coefficient of friction, as may be seen in equation (4). It is already known for the tobacco mosaic virus that the true coefficient of friction is equal to just twice the coefficient of friction of the hypothetical sphere. Thus, one can substitute into the sedimentation equation (4) for the value of the true coefficient of friction, twice the value it would be if the particle were an unhydrated sphere. This latter coefficient of friction is known by Stoke's law to be a function of the radius the particle would have if it were a sphere. The mass of the particle is a function of the radius and the known partial specific volume. Therefore, through the use of an equation similar to (5), it is possible to solve in terms of the hypothetical particle radius. From this value it is simple to calculate the molecular weight. Here again the actual dimensions of the particle can be settled by solid geometry. We have here, then, a method of determining the dimensions of a particle from partial specific volume, viscosity and sedimentation.

In Table III, the results of all these methods are presented along with the values obtained directly with the electron microscope and X-ray diffraction.

TABLE III

THE DIMENSIONS OF TOBACCO MOSAIC VIRUS PARTICLES

Methods	Diameter (m)	Length (m)	Mol.Wt. ($\times 10^{-7}$)
Sedimentation and viscosity	13.6	276	3.32
Sedimentation and diffusion	13.8	256	3.16
Viscosity and diffusion	14.0	283	3.60
Electron microscope and X-ray	15.2	270	4.0

It can be seen that, allowing a reasonable margin of error, all of the methods give essentially the same value for the length and thickness of the tobacco mosaic virus particle, and these values agree with those obtained by direct methods. All of these considerations are complicated, however, by the necessity of assuming the absence of hydration. Preliminary evidence recently obtained by the author and associates makes it appear quite likely that tobacco mosaic virus is hydrated to at least the extent of other viruses which have been studied. The X-ray data of Bernal and Fankuchen show that the distance between tobacco mosaic virus rods oriented parallel in a gel varies exactly with the reciprocal of the square root of the virus concentration. This result

means that any hydration of tobacco mosaic virus must cause swelling in only the two dimensions perpendicular to the length of the particle. When this fact is taken into account, it can be shown that the agreement between the dimensions obtained by direct means and those computed from viscosity and sedimentation data is not affected adversely. However, the agreement of the values calculated from diffusion and either sedimentation or viscosity data with the value obtained by direct means no longer is satisfactory.

Stoke's law for the friction coefficient of a spherical particle moving in a viscous medium was derived for the case of a particle in an infinitely dilute solution. Stoke's law predicts that the coefficient of friction of the particle is directly proportional to the viscosity of the solvent. In actual practice, one always measures the movement of particles in a solution of finite concentration. It is necessary, then, to make a decision in applying the sedimentation equation whether the viscosity of the solvent or the viscosity of the solution should be utilized. It has been the custom to use the viscosity of the solvent. This is the equivalent of making the assumption that the sedimenting particle moves through pure solvent rather than through a solution of similar particles. On the basis of such simple theoretical considerations, one might expect that the sedimentation rate of a protein would be independent of the concentration of protein. This, however, is not at all the case. As shown in Figure 18, when one measures the sedimentation rate of tobacco mosaic virus in solutions of different concentration, one finds that the sedimentation rate does depend upon the concentration of the solution.

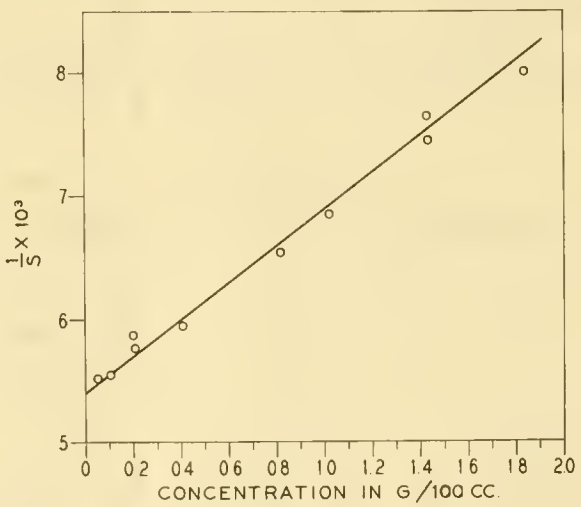


FIGURE 18 - RECIPROCAL OF SEDIMENTATION RATE OF TOBACCO MOSAIC VIRUS PLOTTED AS A FUNCTION OF VIRUS CONCENTRATION. (M.A.Lauffer, J. Am. Chem. Soc. 66, 1195 (1944)).

The reciprocal of the sedimentation constant corrected to water at 20°C is plotted against the virus concentration. A linear relationship is obtained. This concentration dependence of the sedimentation rate is quite a common phenomenon. It is particularly marked in the case of those materials which have a high intrinsic viscosity, such as solutions of gelatin and other proteins, and solutions of high polymeric natural products and synthetic materials. Now, in the case of the tobacco mosaic virus, it turns out that when one plots the viscosity of a solution against the concentration of virus, one also obtains a linear relationship over a considerable concentration range. This result is illustrated in Figure 19.

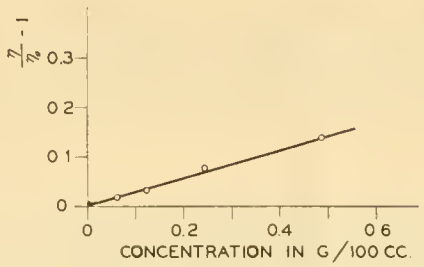


FIGURE 19 - SPECIFIC VISCOSITY OF TOBACCO MOSAIC VIRUS PLOTTED AS A FUNCTION OF VIRUS CONCENTRATION.

It was observed that the slope of this second linear relationship was almost the same as that of the first. This observation suggested that the dependence of the sedimentation rate of tobacco mosaic virus upon concentration was the same sort of phenomenon as the dependence of the solution viscosity upon concentration and that, therefore, if one corrected the sedimentation rate of the virus for the viscosity of the virus solution, one would obtain a value which is essentially independent of virus concentration. This is the equivalent of assuming that the virus particle sediments through a virus solution rather than through the solvent. When the sedimentation rate of the virus is corrected for the viscosity of the virus solution, a value for the sedimentation rate is obtained which is essentially independent of virus concentration. This result is illustrated by the data in Table IV.

TABLE IV

THE SEDIMENTATION CONSTANTS CALCULATED IN THE USUAL MANNER AND THOSE CORRECTED FOR SOLUTION VISCOSITY OF TOBACCO MOSAIC AT VARIOUS CONCENTRATIONS

Concentration (g./100 cc.)	s^0_{20} (Svedberg units)	s^0_{20}
0.05	181	184
.102	180	185
.20	173	183
.20	170	180
.20	170	180
.20	170	180
.204	173	183
.408	168	188
.816	153	189
1.02	146	189
1.43	134	188
1.43	131	184
1.84	125	191

A review of the literature showed that the dependence of sedimentation rate upon concentration for a great variety of materials was reduced substantially when correction was made for the actual viscosity of the solution. These results tempt one to formulate as a working hypothesis that the displacement

of a particle through a viscous medium involves in its hydrodynamic interaction the solution as a whole rather than merely the solvent. Whether this hypothesis is right or wrong makes relatively little difference. It should, in either case serve as a stimulus for the further investigation of hydrodynamic interaction and should, therefore, provide useful service to physical chemistry.

Influenza Virus

The size and shape of influenza virus has also been investigated in considerable detail. A particular preparation was subjected to electron microscopy, ultracentrifugation and viscosity studies by Dr. Stanley and myself. The micrograph reproduced in Figure 20 is of this preparation.

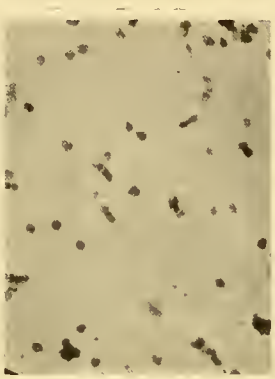


FIGURE 20 - ELECTRON MICROGRAPH OF PR 8 INFLUENZA A VIRUS X12,000. (M.A. Lauffer and W.M. Stanley, J. Exp. Med. 80, 531 (1944)).

The magnification is about 20,000. From the original photograph, whose magnification factor was known accurately, it was possible to measure both the size and distribution of sizes of the spherical particles. The mean diameter of these spherical particles was found to be about 116 millimicrons. These particles are not all of exactly the same size. Figure 21 shows the distribution of particle sizes.

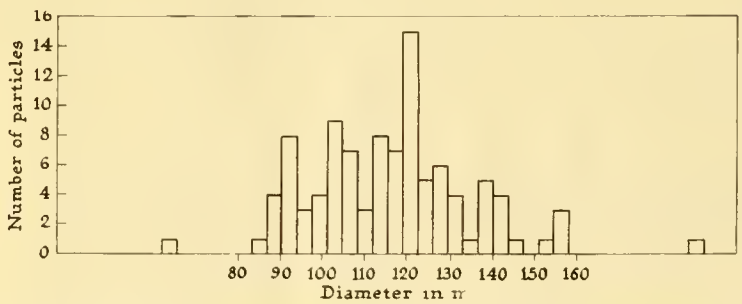


FIGURE 21 - DISTRIBUTION OF DIAMETERS OF PR 8 INFLUENZA A VIRUS PARTICLES

A more or less continuous distribution can be seen. From this, it is possible to calculate that the standard deviation of the distribution of particle diameters is 15% of the mean diameter. With the electron microscope it has been possible to learn something of the external physical nature of influenza virus particles, but little was learned about the internal structure. For this, it is necessary to turn to the ultracentrifuge.

In Figure 22 the results of an ultracentrifugation experiment on influenza virus are shown.

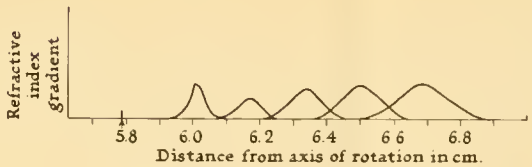


FIGURE 22 - TRACINGS OF SEDIMENTATION DIAGRAMS OBTAINED BY SCHLIEREN METHOD WITH PR 8 INFLUENZA A VIRUS. (M.A.Lauffer and W.M. Stanley, J. Exp. Med. 80, 531 (1944)).

The boundary between virus solution and solvent is represented by a peak. The center of the peak tells where the center of the boundary is at a given time and the sharpness of the peak tells how foggy the boundary is. The successive peaks represent the boundary at five minute intervals after the experiment was started. From the displacement of the peak centers and the speed of the centrifuge, the sedimentation constant can be calculated to be 722×10^{-13} cm. per second per unit field, or 722 Svedberg units. From the progressively greater breadth of the curves, it is possible to determine the degree of boundary fogging, and from that to get an idea of the degree of inhomogeneity of the virus. The partial specific volume is 0.79 cc/grams. This corresponds to a dry density of 1.26 g/cc. With this figure and the value of the sedimentation constant, it is possible to calculate, using equation (6), that the diameter of the influenza virus particle is 70 millimicrons. This must be compared with the value of 115 obtained with the electron microscope. Obviously something is wrong! The difficulty could be attributed to either of two possible causes. First, the assumption that the particles are spheres could be wrong. However, this is unlikely in view of the results obtained with the electron microscope. Second, the density of the particles in solution may not be as great as in the dry state. That is, the influenza particle in suspension might soak up a certain amount of water, thereby increasing its size and decreasing its density. This idea seems plausible, and it can be tested by using the ultracentrifuge.

The rate of sedimentation of a particle suspended in a solvent is directly proportional to the differences between the density of the particle and the density of the solvent. This follows from equation (6) or from a slightly rearranged form of equation (10). Thus, if influenza virus particles were suspended in a solvent with density equal to that of the virus, they would fail to sediment, no matter how fast the centrifuge was run. Also, if the particles were suspended in a medium more dense, sedimentation would take place in a reverse direction, just as in the case of cream in a separator. In fact, if the sedimentation of the particles is measured in several solvents of different densities, one should get a straight line when the sedimentation rate is plotted against the density of the medium, provided the particle has the same density in all the media. Figure 23 presents the results of an experiment in which influenza virus was dissolved in sugar solutions of various densities and then studied in the ultracentrifuge.

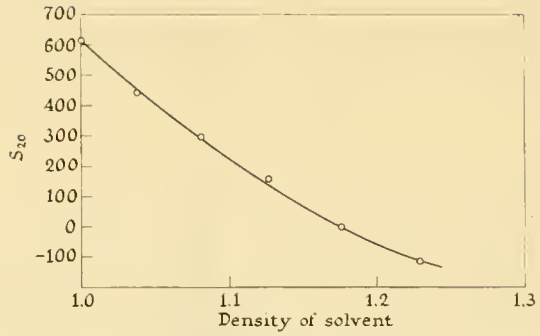


FIGURE 23 - SEDIMENTATION RATES IN SVEDBERG UNITS OF PR 8 INFLUENZA A VIRUS IN SUCROSE SOLUTIONS OF DIFFERENT DENSITIES. (M.A. Lauffer and W.M. Stanley, J. Exp. Med. 80, 531 (1944)).

Here is plotted the sedimentation rate of the virus against the density of the solvent. When the density of the solvent is less than 1.18, the virus sediments toward the periphery of the centrifuge, in a solvent with a density equal to 1.18, it does not sediment at all, and in solvents more dense than 1.18, it moves in a negative direction - toward the axis. This experiment shows quite clearly that the density of a virus particle is 1.18 when it is suspended in a sugar solution with a density of 1.18. It must be remembered that in the dry state, the density of the virus particle is 1.26. Therefore, in solution the virus must contain some water.

There is something peculiar about these results, however. If the density of the virus particle remains constant, one should get a straight line when sedimentation rate is plotted against solvent density. But here a curved line was obtained. This is evidence that the density of influenza virus is not a constant, but changes as the sugar content of the surrounding medium changes. This suggests that, in solvents containing very little sugar, the density of the virus is even less than 1.18. In other words, in the presence of low sugar concentrations, the virus contains more water than in the presence of high sugar concentrations. The density of the virus particle in a solvent free of sugar can be estimated by drawing a straight line tangent to the curve at the point representing zero concentration. This line will intersect the zero level of sedimentation rate at a point corresponding to the density of the sugar solution in which the virus would float if the virus did not increase in density as the sugar concentration increases. One finds a value of about 1.1, showing that the density of the virus in a solvent free of sugar is about 1.1. A partial reason that the virus density increases as the sugar content is increased is obvious. The sugar solution has a high osmotic pressure, and it accordingly draws water out of the virus. The higher the sugar content, the more water is drawn out. A more accurate way of determining the density of the virus in solution is to utilize a material for varying the density of the medium which does not affect the osmotic pressure. Sharp and other associates of Beard at Duke University utilized a protein, serum albumin, for this purpose. Their data are shown in Figure 24.

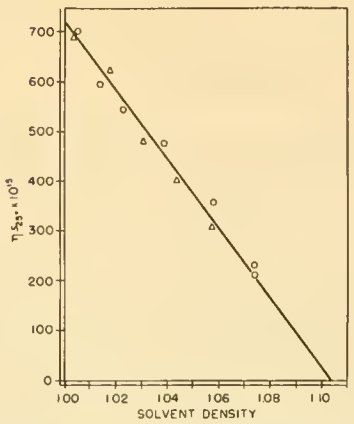
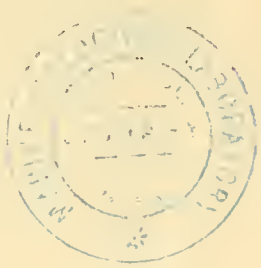


FIGURE 24 - SEDIMENTATION RATE VERSUS SOLVENT DENSITY FOR PR 8 INFLUENZA A VIRUS IN SERUM ALBUMIN SOLUTIONS. (D.G. Sharp, et al, Science 100, 151 (1944)).

Here sedimentation rate of influenza virus is plotted against solvent density. This time the data fall on a straight line. They indicate that in a solvent of density 1.10 the virus would not sediment. Therefore, the hydrated density of influenza virus is 1.10. This result is accurate to at least one decimal place more than the one obtained from the study using sugar solutions. It clinches the argument that influenza virus contains an appreciable amount of water when it is suspended in an aqueous medium. From the sedimentation constant and the wet density of 1.10, it is possible to calculate that the diameter of the wet influenza particle is 114 mu. Also, from the wet density, the dry density, and the density of water, it is possible to calculate that the wet influenza virus particle contains 60% by weight of water.

There are several general conclusions that can be drawn from these studies on the size and shape of viruses. First of all, viruses, particularly tobacco mosaic virus, provided a remarkable tool for investigation for the first time of the validity of theoretical equations derived from hydrodynamic considerations relative to the size and shape of suspended particles. Thus, the study of viruses has contributed to the advancement of physics and physical chemistry. Second, many viruses contain water in their make-up. The measurement of the amount of water associated with the viruses represents the first unambiguous determination of the water of hydration of any protein in solution. Third, influenza virus was demonstrated not to be strictly uniform in size. Thus, this virus does not satisfy the criterion demanded of molecules. In general, most viruses causing diseases of animals and man thus far studied seem to resemble influenza virus more than plant viruses.



CHAPTER III

DISINTEGRATION OF VIRUSES

Introduction

Virologists and bacteriologists have long recognized that some viruses and also some bacteria are much more stable to heat than others. In fact, the thermal death point has come to be recognized as one of the important characteristics of such agents. It was thought, at one time, that the thermal death point was a specific temperature below which the virus was stable and above which it became inactivated or killed. However, in more recent years, biologists have come to realize that in reality no such thing as a thermal death point exists. The inactivation of a virus is now recognized as a process which goes on at all temperatures but which, like most chemical reactions, proceeds more rapidly at high temperatures than at low temperatures. In view of this concept, the thermal death point, or the thermal inactivation point, merely becomes that temperature at which the rate of inactivation is sufficiently high so that most of the activity is destroyed during the arbitrary period of time chosen for the experiment. It is evident, under these circumstances, that the concept of a thermal death point or thermal inactivation temperature is not as useful as specific knowledge concerning the way in which inactivation rate varies with the temperature. The question of thermal stability of viruses is thus reduced to a problem in kinetics. It has been shown through many studies that the destruction of biological activity can be thought of as being one phase of the more general problem of denaturation of proteins. In view of this background, it was thought worthwhile to investigate not only the inactivation but also the disintegration or denaturation of virus proteins. Tobacco mosaic virus and influenza virus have been studied in greatest detail. The remainder of this chapter will consist of an examination of some of the results obtained in these studies.

Tobacco Mosaic Virus

It has been known for several years that the ability of tobacco mosaic virus to infect plants could be destroyed by heating at rather high temperatures, between 90° and 100° C. After tobacco mosaic virus was isolated and purified by Stanley, it became possible to study the denaturation of tobacco mosaic virus protein at high temperatures. The unfortunately ambiguous term, denaturation, will be defined for the purpose of the present discussion, as the changes that take place when a protein capable of being dissolved in neutral salt solutions is transformed into a state in which it is no longer soluble in such solvents. When a neutral solution of tobacco mosaic virus is heated to 80° or 90° C., virus infectivity is lost and a curdy precipitate forms gradually and does not redissolve upon cooling. This insoluable material has a molecular weight, not of thirty million, but of less than one hundred thousand. The kinetics of the denaturation of tobacco mosaic virus was studied in cooperation with Price at the Rockefeller Institute. This was done by measuring chemically the amount of tobacco mosaic virus remaining in solution after various periods of heating at known temperatures. The results of a typical experiment are shown in Figure 25.

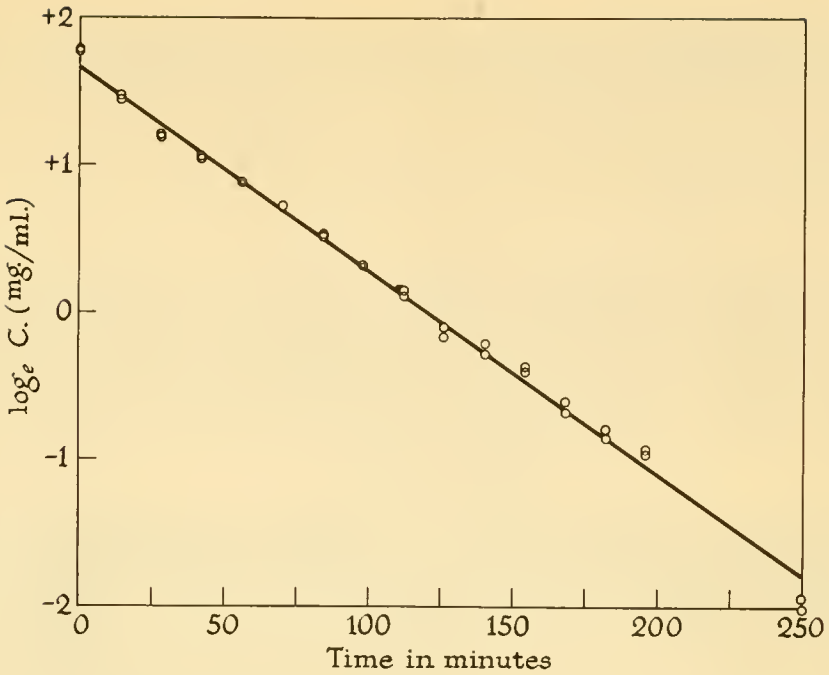


FIGURE 25 - LOG CONCENTRATION OF TOBACCO MOSAIC VIRUS PROTEIN PLOTTED AS A FUNCTION OF TIME OF HEATING AT 69.8° C. in $\frac{M}{10}$ PHOSPHATE BUFFER AT pH 7. (M.A.Lauffer and W.C. Price, J. Biol. Chem. 133, 1(1940)).

Here is plotted the natural logarithm of the concentration of tobacco mosaic virus remaining in solution against the time in minutes of heating at about 70° C. It can be observed that the data fall reasonably well upon a straight line. This type of concentration-time relationship is that characteristic of reactions of the first order. A first order reaction is one in which the rate of decrease of concentration of the reactant is proportional at every instant to the concentration of the reactant present at that instant. This fact can be expressed most eloquently by the simple differential equation

$$\frac{d[V]}{dt} = k[V] \qquad (1)$$

where $[V]$ is the virus concentration and t the time. Upon integration, equation (1) becomes

$$\log_e [V] = \log_e [V_o] - kt \qquad (2)$$

and it is in this form that a straight line can be obtained upon plotting. In both of these equations, the constant k is the specific reaction velocity for the reaction under study. It can be defined as the rate of the reaction at the instant that the concentration of the reactant is unity. From the integrated form of the equation, it is obvious that the constant k is simply the slope of the line obtained when the natural log of concentration is plotted against time.

There are several things worth knowing about the kinetics of the denaturation of any protein. First of all, one should decide with certainty whether or not the process is really a reaction of the first order. It is evident from a consideration of the data in Figure 25 that there is a certain amount of error

associated with this experiment. Those who know how hard it really is to differentiate zero, first and second order reactions with systems exhibiting any appreciable error might wonder whether the data here seen would not fit the graph of some other kinetic process as well as that here indicated. These data were subjected to an appropriate statistical procedure. It was found that an equation of a first order reaction was overwhelmingly favored. On can then say with practical certainty that heat denaturation of tobacco mosaic virus is a reaction of the first order.

The next issue of importance is to observe in just what manner the specific rate, the k in equations (1) and (2), varies with the temperature. It is easier to understand the significance of this sort of observation if we have in mind some simple theoretical picture of the mechanism of virus denaturation. It is consistent with present day concepts of reaction kinetics to imagine that, for a virus molecule or particle to become denatured it must first pass into an activated state. This reaction can be symbolized by the following equation:

$$V \underset{}{\overset{K}{\rightleftharpoons}} V^* \xrightarrow{k'} nD \qquad (3)$$

V represents a unit of virus in the normal state, V* represents a unit of virus in the activated state, nD represents n units of denatured virus, K is an equilibrium constant, and k' is a rate constant. The activated state is assumed to be in reversible equilibrium with the virus in its ordinary state. However, during the short period of time that any one virus particle is in the activated state, there is a certain probability, k', that it will disintegrate or change over into denatured virus. The rate at which the activated virus particle disintegrates can be thought of as being proportional to the concentration of activated virus at the moment. In reality the rate of change of activated virus into denatured material is the rate of change of ordinary virus into denatured material. Therefore the rate of change of ordinary virus into denatured material is proportional to the concentration of virus in the activated state. Since the activated virus and the normal virus are in equilibrium, the concentration of virus in the activated state is equal to the equilibrium constant times the concentration of virus in the normal state. This statement can be only approximately true. It would be more accurate to say that the thermodynamic activity of the virus in the activated state is equal to the equilibrium constant times the thermodynamic activity of the virus in the normal state. On the basis of this reasoning, it can easily be seen that the rate of denaturation of the virus is proportional to the virus concentration and the equilibrium constant for the reaction: normal virus yields activated virus. In order to determine how the rate of denaturation of virus varies with the temperature or with any other variable, all one needs to determine is how this equilibrium constant varies with the variable under consideration. These considerations can be summarized by a few equations.

$$\frac{d[V]}{dt} = -\frac{d[V^*]}{dt} = k'[V^*] \qquad (4)$$

But, $\frac{[V^*]}{[V]} = K$; more accurately, $\frac{f^*[V^*]}{f[V]} = K$

Therefore, $$-\frac{d[V]}{dt} = k' \; K[V] \; f/f^* \qquad (5)$$

By comparing (1) and (5), $$k = k' \; K \; f/f^* \qquad (6)$$

and $$\ln k = \ln k' + \ln f/f^* + \ln K = \text{const} + \ln K \qquad (7)$$

The brackets indicate concentration, f is the activity coefficient of normal virus, and f* is that of virus in the activated state. The problem of

determining the manner in which the rate constant k changes with temperature reduces to a determination of the change in the equilibrium constant with temperature. Two relationships derived from elementary thermodynamics should be recalled:

$$-\Delta F^\circ = RT \ln K \tag{8}$$

and

$$\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ \tag{9}$$

Therefore,

$$-\Delta H^\circ + T\Delta S^\circ = RT \ln K$$

$$\text{and} \quad \ln K = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{RT} \tag{10}$$

By combining equations (7) and (10), one obtains:

$$\ln k = \text{const} + \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{R}\frac{1}{T} \tag{11}$$

ΔF°, ΔS° and ΔH° are the standard free energy, standard entropy change, and standard heat of reaction respectively. R is the gas constant and T is the absolute temperature. Thus, when $\ln k$ is plotted against $\frac{1}{T}$, a straight line should be obtained. Its slope should be $-\frac{\Delta H^\circ}{R}$, where ΔH° is the standard heat of the reversible reaction $V \rightleftharpoons V^*$. This is sometimes called the energy of activation.

The thermal denaturation of tobacco mosaic virus was studied at several different temperatures and the specific reaction velocity for each was obtained. Then the log of K was plotted against the reciprocal of the absolute temperature, as is seen in Figure 26.

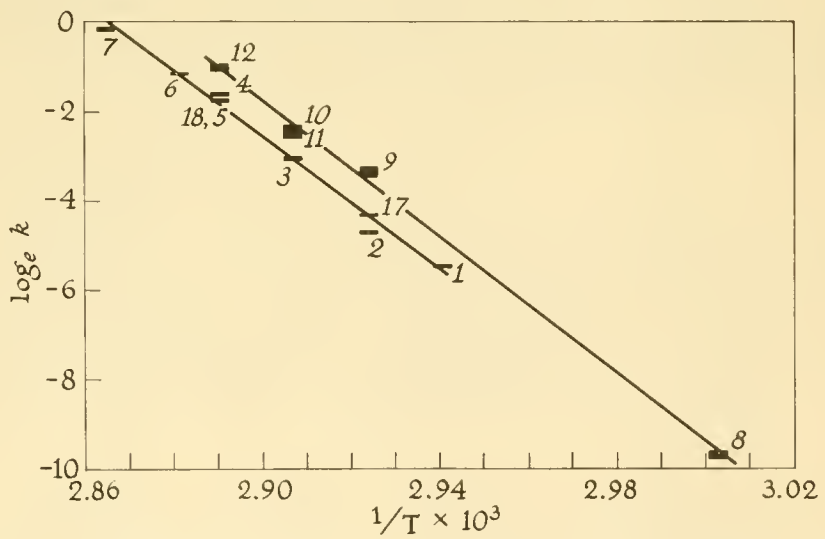


FIGURE 26 - VELOCITY CONSTANTS FOR THERMAL DENATURATION OF TOBACCO MOSAIC VIRUS PROTEIN AT pH 7 PLOTTED AS A FUNCTION OF ABSOLUTE TEMPERATURE ACCORDING TO THE ARRHENIUS EQUATION: UPPER CURVE FOR SYSTEMS WITH INITIAL VIRUS CONCENTRATION OF 3 mg/cc; LOWER CURVE FOR SYSTEMS WITH INITIAL VIRUS CONCENTRATION OF 6 mg/cc. (M.A.Lauffer and W.C. Price, J. Biol. Chem. 140, 509 (1941)).

By confining attention to the upper curve, one can see that a straight line actually is obtained when the data are thus plotted, at least over the temperature range of 60° to 76° C. From the slope of this line, the energy of activation for the thermal denaturation of tobacco mosaic virus was calculated to be 153,000 calories per mole. This value is significant because it is very high; it is similar to the energies of activation for other protein denaturations and much higher than the energy of activation for most ordinary chemical reactions. There is one more thing that must be observed on this figure. All of the data fitting the lower line were obtained with virus solutions at an original concentration of 6 mg. per cc., and those fitting the top line were obtained with virus at an original concentration of 3 mg. per cc. These results, when transformed from logs back to simple numbers, show that a virus solution of 3 mg. per cc. initial concentration will denature with a specific reaction velocity about twice that of a virus solution at an initial concentration of 6 mg. per cc. Now, the specific reaction velocity k of any process, is supposed to be a constant which is entirely independent of the initial concentration of the reactant. Hence this observation constitutes an exceptional case and is, therefore, worth some attention, because it must mean something. Later in this discussion, this matter will be considered more fully. At the present it should be pointed out that this observation affords a chemical basis for the earlier observation of Price: that the stability of heat of tobacco mosaic and other plant viruses decreases with dilution of the virus. It is interesting to extrapolate the data of Figure 26 to room temperature to see how fast tobacco mosaic virus would denature. When one does this, he finds that 30° C., log k = - 33.15. Transforming to numbers, $k=4 \times 10^{-15}$ min.$^{-1}$ at 30° C. This means that the virus should have a half life of around a billion years.

It has been known for many years that some viruses can be destroyed by subjecting them to extremely high pressures. In 1941, Dow cooperated in some experiments on the denaturation of tobacco mosaic virus at high pressures. It was found that, when the virus was exposed to pressures between 5,000 and 10,000 atmospheres or kilograms/sq.cm., it was transformed into an inactivated coagulum. Nucleic acid was split off in the process. The kinetics of the reaction at a pressure of 7500 kilograms per square centimeter was studied in a little detail. The results are shown in Figure 27.

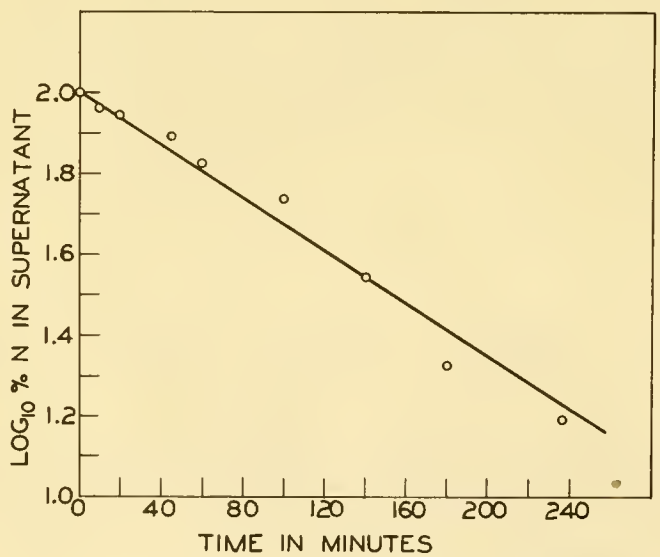


FIGURE 27 - LOG CONCENTRATION OF TOBACCO MOSAIC VIRUS PROTEIN PLOTTED AS A FUNCTION OF TIME OF EXPOSURE TO PRESSURE OF 7500 KILOGRAMS/Sq.CM. AT 30° C. (M.A. Lauffer and R.B. Dow, J. Biol. Chem. 140, 509 (1941)).

It was found that, when the logarithm of the amount of nitrogen in the supernatent fluid, which is proportional to the amount of virus remaining undenatured is plotted against the time, a straight line is obtained. This would indicate that the pressure denaturation of tobacco mosaic virus protein is a reaction of the first order. From the data obtained, one can calculate that the specific reaction rate at 30° C. is 7.45×10^{-3} min.$^{-1}$. The logarithm to the base e of that figure is a -4.9. It was estimated previously that, at 30° C. at a pressure of 1 atmosphere or about 1 kilogram/sq.cm., the extrapolated value of the reaction rate is 4×10^{-15}. Thus the value at a pressure of 7,500 kg/sq.cm. is about 10^{12} as fast.

It might be asked, by what mechanism does an increase in pressure cause such a tremendous increase in rate? If one examines the theory again, one can see that an increase in pressure might cause a shift in the equilibrium between normal virus and activated virus. If the activated virus particle has a slightly lower molecular volume than the normal particle, then an increase in pressure would shift the equilibrium toward more activated virus. This would speed up the reaction. This idea can be treated quantitatively through the use of some of the results of thermodynamics. Students of elementary thermodynamics learn that the following equation holds:

$$\left(\frac{d\,(\Delta F^\circ)}{dP}\right)_T = \Delta\bar{V} \qquad (12)$$

$\Delta\bar{V}$ is the change in partial molar volume accompanying a reaction. By combining (7), (8), and (12), one obtains

$$\frac{d \ln K}{dP} = -\frac{\Delta V}{RT} = \frac{d \ln k}{dP} \qquad (13)$$

If the data described above are substituted into equation (13), a value of -97,000 cc. is obtained for $\Delta\bar{V}$. This means that in the process of passing from the normal state to the activated state, there is a decrease in volume of 97,000 cc. per mole. The molecular weight of tobacco virus is about 30-35,000,000 grams and the specific volume is about 0.75 cc/gram. Therefore, the molar volume of the virus is about 25,000,000 cc. The shrinkage in passing to the activated state is thus about 1 part in 250, or 0.4%.

When tobacco mosaic virus is dissolved in a 6 molar solution of urea buffered to pH 7 with phosphate buffer, many changes take place simultaneously, as illustrated in Figure 28. Virus infectivity is lost. The virus protein with a sedimentation constant of 185×10^{-13} is gradually converted into components with sedimentation constants ranging from 50 to 2×10^{-13}. These smaller particles, although completely soluble in urea, come out of solution when the urea concentration is reduced by dilution or by dialysis. The precipitated material is free of nucleic acid.

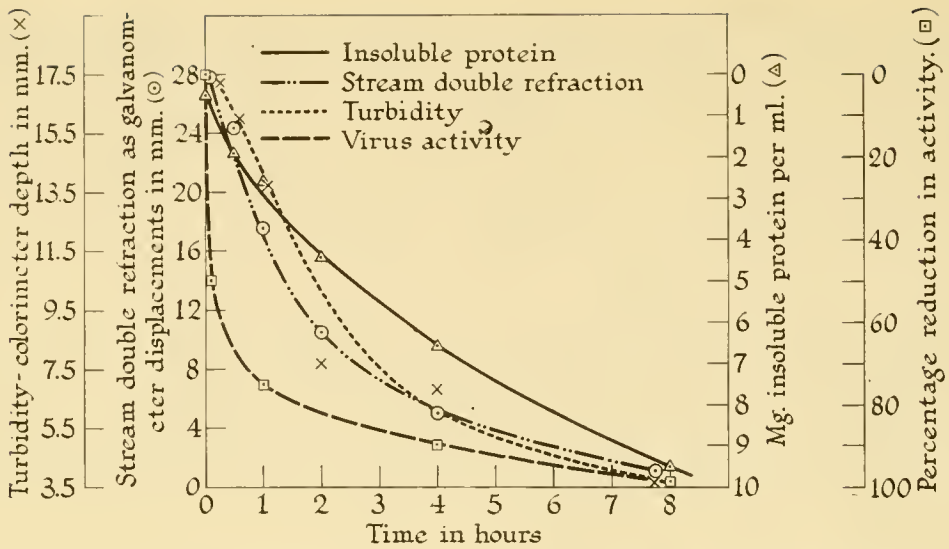


FIGURE 28 - DECREASE IN SOLUBILITY, STREAM DOUBLE REFRACTION, TURBIDITY AND INFECTIVITY OF TOBACCO MOSAIC VIRUS, PLOTTED AS A FUNCTION OF TIME OF EXPOSURE AT ROOM TEMPERATURE TO 6M UREA. (W.M. Stanley and M.A. Lauffer, Science 89, 345 (1939)).

Accompanying this breakdown is a gradual appearance of free sulfhydryl groups as indicated by the nitroprusside test. These chemical changes are paralleled by certain physical changes: decrease in the ability to show stream double refraction characteristic of tobacco mosaic virus solutions, and decrease in the turbidity or opalescence of the material. The course of the reaction can be followed by measuring the decrease in the amount of protein soluble in dilute electrolyte solutions using standard chemical procedures. This is certainly the most foolproof method, but also the most tedious. The reaction can be followed much more easily by measuring the decrease in turbidity of the virus, using a photoelectric colorimeter. Normal active virus in urea solution as in water, is opalescent. The completely denatured material is water-clear in urea. Some sacrifice in reliability might result from the adoption of this technique, but the advantage gained from the much greater convenience more than compensates.

As in the other denaturation processes, particular attention was paid the kinetics of the reactions involved. In Figure 29 it can be seen that the log of the amount of protein remaining undenatured decreases linearly with time when tobacco mosaic virus is dissolved in 6M urea.

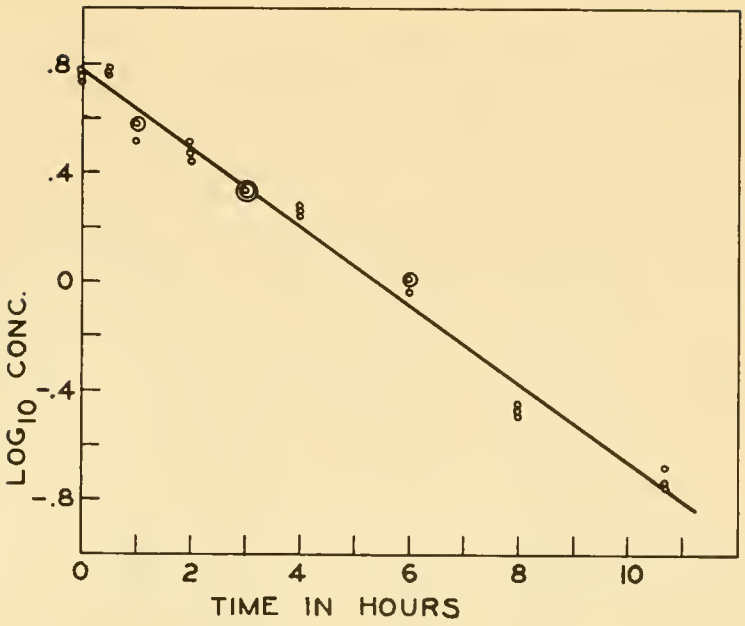


FIGURE 29 - LOG CONCENTRATION OF TOBACCO MOSAIC VIRUS PROTEIN PLOTTED AS A FUNCTION OF TIME EXPOSURE TO 6 M urea. (M.A. Lauffer, J.A.C.S. 65, 1793 (1943)).

The reaction was followed chemically in this case. This would indicate that urea denaturation of tobacco mosaic virus, in common with heat denaturation, is a process of the first order. Again statistical procedures were used to demonstrate that the fit of these data to the equation of a first order reaction is overwhelmingly better than to the equations of any other reaction orders commonly encountered. Just as in the case of the thermal denaturation of tobacco mosaic virus, the specific reaction rate for the urea denaturation under a fixed set of conditions can be calculated from the slope of the line obtained when the data are plotted in the manner here illustrated, whether the data are obtained by chemical or turbidimetric methods.

A series of experiments was carried out to determine the influence of several variables upon the magnitude of the specific reaction velocity. Because of the convenience, the turbidimetric method was used throughout the following experiments. In the first stages of this work, Stanley and I discovered that urea denaturation of tobacco mosaic virus proceeded faster at 0° C. than at room temperature, but also faster at 40° C. than at room temperature. Bawden and Pirie later confirmed this unusual behavior. More recently, the more extensive data presented in Figure 30 have been obtained. The specific reaction velocity is plotted against the absolute temperature. The U-shaped relationship obtained is obviously a very unexpected behavior, quite contrary to the sense of usual rate theory which requires that reaction velocities decrease steadily as temperature decreases. However, that this condition may be general for urea denaturation of proteins is indicated by the few reports available in the literature. Hopkins in 1930 reported that the denaturation of egg albumin by urea has a negative differential rate temperature coefficient over the temperature range 0 to 37° C. More recently, Clark reported a positive differential rate temperature coefficient for egg albumin in urea. In 1938 Diebold and Juhling showed that fibrinogen has a positive temperature coefficient of denaturation in urea. In 1939 Drabkin reported that carboxy hemoglobin also has a positive temperature coefficient of urea denaturation over the temperature range 20 to 38° C. It seems likely that in each of these cases only one horn of the temperature rate function was being investigated.

The unprecedented behavior of tobacco mosaic virus can be explained on the basis of a few simple and plausible assumptions.

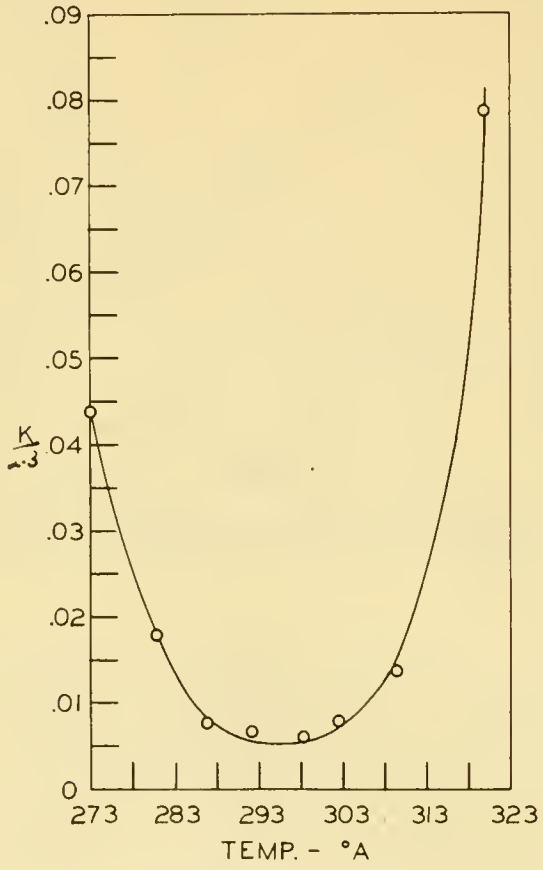


FIGURE 30 - SPECIFIC REACTION VELOCITY IN MIN.$^{-1}$ OF UREA DENATURATION OF TOBACCO MOSAIC VIRUS PLOTTED AS A FUNCTION OF ABSOLUTE TEMPERATURE. (M.A. Lauffer, J. Amer. Chem. Soc. 65, 1793 (1943)).

The curve in Figure 30 could be visualized as being made up of the sum of two curves, one of which decreases from a high value and approaches zero assymptotically as temperature is increased towards room temperature, and the other of which does the same thing as temperature is decreased towards room temperature. Each of these curves could represent a separate denaturation process, one with a negative temperature coefficient and one with a positive coefficient. Let us, therefore, make the assumption that tobacco mosaic virus in urea can be denatured by at least two simultaneous parallel routes or processes. It is possible to conceive of an urea denaturation process with a negative differential temperature coefficient, if it is assumed that the urea must react reversibly with the virus to form an urea-virus compound, which then can denature according to the usual laws. If this mechanism is right, the rate of change of native virus to denatured virus by this process must depend at all times upon two things: (1) the specific rate for disintegration of the complex and (2) the fraction of the total undenatured virus in the combined state. These ideas can be represented by the following equation:

$$xU + 1\,V \underset{}{\overset{K'}{\rightleftharpoons}} V\,U_x \underset{}{\overset{K''}{\rightleftharpoons}} V^* \xrightarrow{k'} nD \qquad (14)$$

U represents a molecule of urea, and K' and K'' are equilibrium constants.

By analogy with the reasoning involved in deriving equation (7), one can deduce that the over-all rate constant k is dependent upon the constant k' , the two equilibrium constants, the urea concentration to the power x, and the appropriate activity coefficient ratio. If the reaction between virus and urea is exothermic, K' will decrease as temperature is increased. K'', on the other hand representing an endothermic reaction, will increase as temperature increases. If the rate of decrease of K' is greater than the rate of increase of K'', the product of the two will decrease as temperature is increased. In other words, this particular urea denaturation process will have a negative differential temperature coefficient. This is only half of the story. It is also necessary to postulate a parallel process with a positive temperature coefficient. Such a process could result from a mechanism similar to that postulated above, excepting that in this case the decrease of K' with temperature must be less than the increase of K'' with temperature. If these considerations are right, then the specific reaction velocity of urea denaturation should be proportional to the sum of two terms, each of which governs a separate process. The assumption that there are at least two mechanisms for the urea denaturation of tobacco mosaic virus, that they both depend upon a reaction between urea and virus to give a complex which is then denatured, and that at least one of these reactions has a positive differential rate temperature coefficient can account for the way in which the overall urea denaturation of tobacco mosaic virus varies in rate with temperature. Both of the postulated mechanisms involve the reaction of urea with virus. One can deduce from this that the reaction rate ought to be proportional to some power of the urea concentration, that is, the logarithm of the reaction rate ought to be proportional to the logarithm of the urea concentration. In Figure 31 are shown results of an experiment in which the reaction rate was measured in urea solutions of different concentrations.

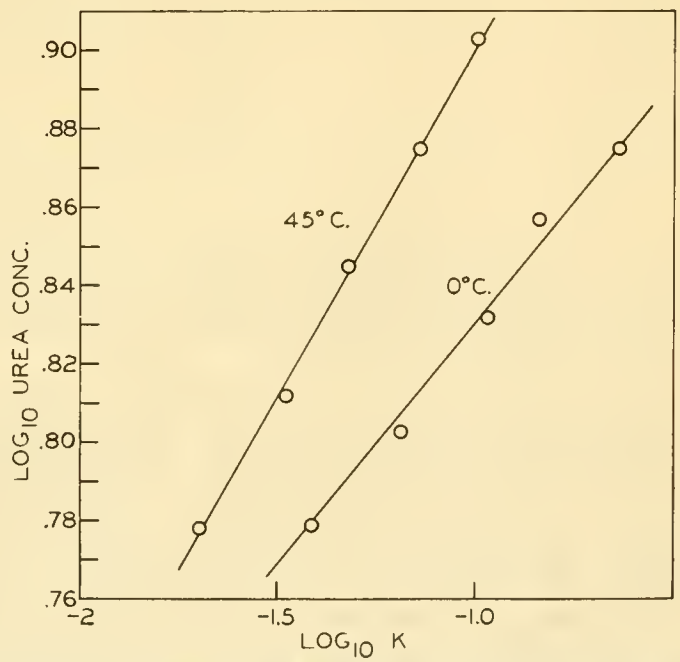


FIGURE 31 - RELATIONSHIP BETWEEN THE SPECIFIC REACTION VELOCITY IN MIN.$^{-1}$ at 0° AND 45° C. OF THE UREA DENATURATION OF TOBACCO MOSAIC VIRUS PROTEIN AND THE MOLAR CONCENTRATION OF UREA. (M. A. Lauffer, J. Amer. Chem. Soc. 65, 1793 (1943)).

Measurements were made at 0°C. and also at 45° C. The logarithm of the reaction velocity is plotted against the logarithm of the urea concentration for each case. It can be seen that the data fall on straight lines, as required by the theory.

It may be recalled that the heat denaturation of tobacco mosaic virus varied, in contradiction to all accepted rules, with the reciprocal of the initial virus concentration. A fairly extensive study of the effect of initial concentration of virus in the urea denaturation was accordingly carried out. In Figure 32 it can be seen that as the initial concentration of virus is decreased, the specific reaction rate for the denaturation increases. This confirms the behavior for thermal denaturation.

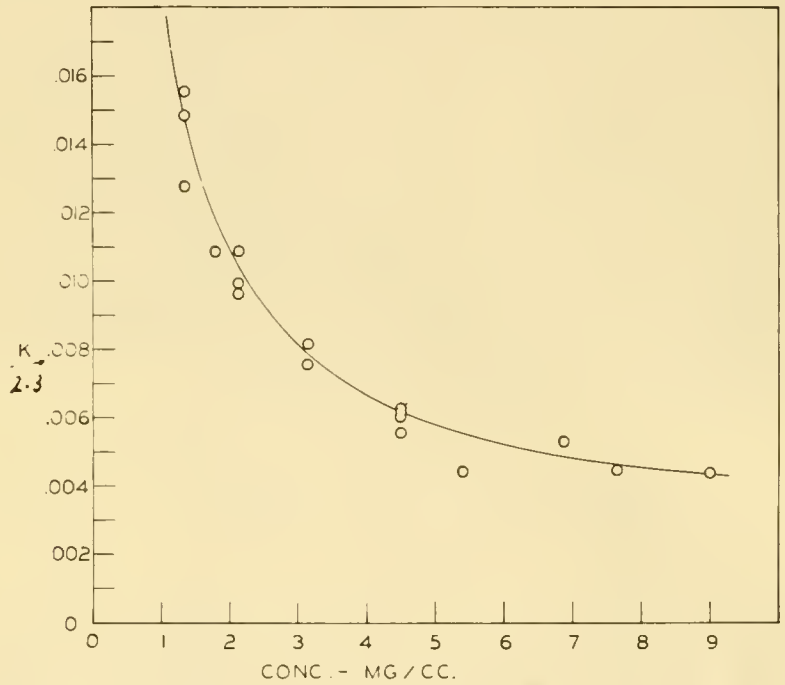


FIGURE 32 - SPECIFIC REACTION VELOCITY IN MIN.$^{-1}$ OF THE UREA DENATURATION OF TOBACCO MOSAIC VIRUS PROTEIN PLOTTED AS A FUNCTION OF INITIAL VIRUS CONCENTRATION. (M.A. Lauffer, J. Amer. Chem. Soc. 65, 1793 (1943)).

If equation (7) is re-examined, it can be seen that the reaction velocity constant is proportional to, among other things, the ratio of the activity coefficient of the intact virus to the activity coefficient of the activated virus. The equation applying to this case would be more complicated than (7), but the principle is the same. It is well known that the activity coefficients of many materials are dependent upon their own concentration. In some cases the mechanism by which the activity coefficient is influenced by concentration is well understood. In other cases this mechanism is not understood. It is conceivable that the activity coefficient ratio is in some way a function of the initial concentration of virus. Such a relationship could account for the variation of the reaction rate with initial virus concentration. It would constitute a formal thermodynamic rationalization of the process. However, it would constitute self-deception to pretend that this really constitutes an explanation of what

is happening. It is merely a familiar trick of the physical chemist to dodge an embarrassing issue.

Influenza Virus

In the remainder of this chapter the inactivation or denaturation of influenza virus will be discussed. Influenza virus has three interesting biological activities. First and foremost is the ability of the virus to infect animals, including man, the mouse, and the chicken embryo. Another interesting property of influenza virus is its ability to agglutinate red blood cells of chickens and of certain other animals. This ability was first discovered by Hirst of the Rockefeller Foundation. It constitutes an extremely useful property of the virus. A third biological activity of influenza virus is the ability to induce specific protecting antibodies in the blood stream of hosts which have been inoculated with the virus or which have been subjected to influenza infections. All of these biological properties seem to be associated with the 100-millimicron spherical particles now accepted as the influenza virus.

The ability to agglutinate red cells can be measured quantitatively with a probable error of something like 10 to 25%. This is done by finding the concentration of virus necessary to agglutinate half of the red cells in a 3/4 or 1% suspension. Since the agglutination ability can be estimated more or less quantitatively, its rate of destruction can be measured more or less quantitatively. The rate of destruction of the ability to agglutinate red blood cells has been studied under a variety of conditions. As can be seen in Figure 33, when a solution of influenza A virus is heated, the reciprocal of the square root of red blood cell agglutinating activity is a linear function of time.

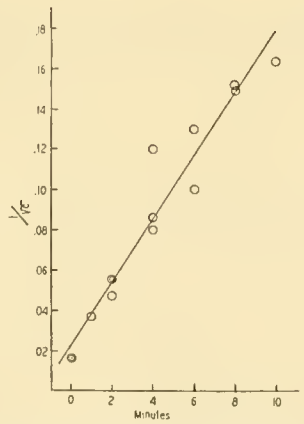


FIGURE 33 - RECIPROCAL OF SQUARE ROOT OF HEMAGGLUTININ ACTIVITY OF PR 8 INFLUENZA A VIRUS PLOTTED AS A FUNCTION OF HEATING AT 61° C. IN PHOSPHATE BUFFER AT pH 6.9 (M.A. Lauffer and H.L. Carnelly, Arch Biochem. 8, 265 (1945)).

This means that the ability to agglutinate is destroyed by heating and that the relationship of the amount remaining to time is the one shown. This is an unexpected complication. One would anticipate that the destruction of hemagglutinin would be a reaction of the first order. Instead, the data shown in this figure indicate that the reaction is of the three halves order. In all probability this apparent order is not real, for a complicated kinetic picture such as this could result from inhomogeneity of the agglutinating factor with respect to rate of destruction. In any case, the linear relationship between the reciprocal of the square root of concentration and the time affords a convenient empirical method of determining the relative rates of the reaction under

various conditions. A sort of reaction velocity constant can be calculated for the reaction by this means. Such velocity constants were determined in a variety of buffers at different temperatures. In Figure 34 the logarithm of the velocity constant is plotted against the reciprocal of the absolute temperature for several of the buffers.

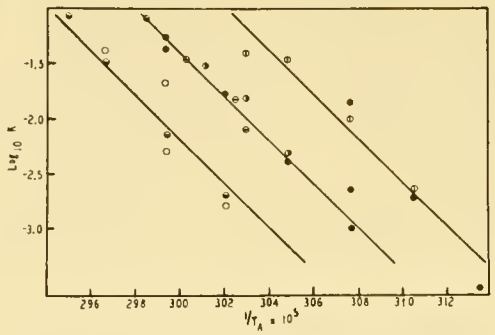


FIGURE 34 - LOG SPECIFIC REACTION VELOCITY OF DESTRUCTION OF HEMAGGLUTININ OF PR 8 INFLUENZA A PLOTTED AS A FUNCTION OF RECIPROCAL OF ABSOLUTE TEMPERATURE. O - Phosphate buffer pH 7.86, O - Phosphate at pH 7.59, O - Phosphate at pH 7.15, O - Phosphate at pH 6.47, O - Ammonia buffer at pH 9.55; O - Phosphate at pH 6.47 (different virus preparation), O - Acetate at pH 5.76. (M.A. Lauffer and E.M. Scott, Arch. Biochem. 9, 75 (1946)).

From these plots the reaction velocity constant at a particular temperature can be read off for each buffer. In Figure 35 the logarithm of the specific reaction velocity at 55° C. for the destruction of hemagglutinin is plotted as a function of pH.

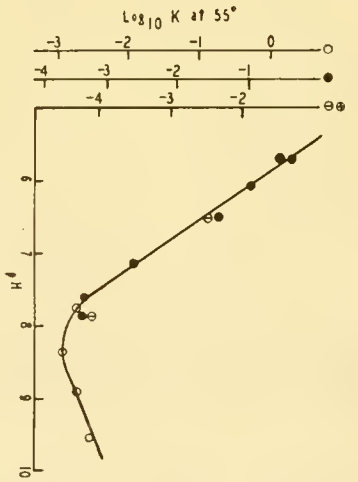


FIGURE 35 - LOG SPECIFIC REACTION VELOCITY AT 55° C. FOR DESTRUCTION OF HEMAGGLUTININ OF PR 8 INFLUENZA A VIRUS PLOTTED AS A FUNCTION OF pH. (M.A. Lauffer and E.M. Scott, Arch. Biochem. 9, 75 (1946)).

It can be seen that the reaction has a minimum rate at pH 8.4. This means that the hemagglutinin has a maximum stability at 55° C. at pH 8.4. It is interesting to observe that the isoelectric point of influenza virus A is at pH 5.4.

The hemagglutinin of the virus is thus at its maximum stability in solutions much more alkaline than the isoelectric point.

Because of the fact that many viruses seem to denature or to become inactivated at rates which vary with the initial virus concentration, it was thought worthwhile to determine whether or not the rate of inactivation of influenza virus hemagglutinin varies with the initial concentration. The data shown in Table V indicate that the reaction velocity for the destruction of hemagglutinin increases as one decreases the concentration of virus.

TABLE V

REACTION VELOCITY CONSTANTS FOR THERMAL INACTIVATION OF CCA OF INFLUENZA A VIRUS

	55°C		58°C		61°C	
V_o	k	kV_o	k	kV_o	k	kV_o
1.00	.00155	.00153	.00110	.00110	.0048	.0048
0.50	.00038	.00027	.00073	.00051	.0033	.0023
0.25	.00227	.00113	.00234	.00117	.0065	.0033
0.12	.00159	.00056	.00248	.00088	.0216	.0076
0.06	.00246	.00061	.00432	.00108	.0117	.0029
0.03	.00309	.00055	.00602	.00106	.0187	.0033

However, it can be seen further that if one multiplies the reaction rate by the square root of the initial virus concentration, one obtains values which do not vary in any orderly manner. This would seem to indicate that the reaction rate is inversely proportional to the square root of initial virus concentration. An explanation for this behavior can be found. As was pointed out previously, the fact that the destruction of hemagglutinin seems to be a reaction of the three halves order, when it should be, in fact, a reaction of the first order, can be explained by the assumption that the hemagglutinin is inhomogeneous with respect to its stability. If one supposes that there are at least two different kinds of hemagglutinin present, that each is destroyed according to a first order reaction, and that the overall destruction of the two or more types is such as to make the reaction appear to be one of the three halves order, then one can show from simple kinetic considerations that the apparent reaction velocity constant calculated on the assumption that the reaction really is of the three halves order will vary with the reciprocal of the square root of the initial total virus concentration. Since this is what does happen, one is justified in considering the dependence of rate of destruction upon the reciprocal of square root of initial concentration as evidence in favor of the complexity of the hemagglutinin.

This question of the destruction of influenza A virus hemagglutinin has its practical aspect. A vaccine has been developed for influenza virus. The vaccine is nothing more than a suspension of the virus which has been rendered non-infectious with formaldehyde. This vaccine is prepared by biological houses and stored until used by a physician. It is very important to know the optimal conditions for storage. What one wants to know is the condition of storage at which the vaccine will lose its ability to form antibodies most slowly. When one studies the rate of destruction of hemagglutinin, one might well provide clues for establishing the conditions most favorable for storage of influenza vaccine.

CHAPTER IV

VIRUSES AS ORGANISMS

The principal reason that man is interested in viruses is that they produce diseases. Thus, one might say that the most important single characteristic of a virus is its ability to produce disease. When a virus infects a host and produces a disease, it invariably multiplies or is multiplied, that is, the number of units of virus is increased enormously. The magnitude of this increase can be appreciated if we consider a few examples. It is possible to infect tobacco plants fairly regularly by rubbing onto the surface of one leaf of such a plant a tobacco mosaic virus solution containing something like 10^{-10} grams of virus per cc. Considerably less than a tenth of a cc is spread over the surface of the leaf. Thus one can say that 10^{-11} grams of virus rubbed onto the surface of a tobacco leaf can cause an infection of the tobacco plant. In a period of just a few weeks, the plant will have developed a systemic virus infection, and then the virus can be isolated from this plant. About one tenth of a gram of virus will be obtained. Thus one can say that from 10^{-11} grams of virus placed on the leaf, 10^{-1} grams of virus can be recovered. This represents a multiplication of 10 billion fold. Actually, this is a minimum multiplication. It is believed that one tobacco mosaic virus rod is the only thing necessary to cause the infection of the plant. Such a virus rod weighs about 10^{-16} grams. The total amount of virus recovered is, as mentioned before, 10^{-1} grams. This would represent a multiplication of 10^{15} fold.

When tobacco necrosis virus is rubbed onto a leaf of a tobacco plant, a necrotic lesion is developed in about sixteen hours. From the size of the lesion and the concentration of virus in the tissue, one can estimate that about 4×10^{-7} grams of virus are contained. This represents about 10 billion virus particles. Since it is believed that all of these virus particles were derived from a single virus particle, it can be deduced that the virus multiplied 10 billion fold in a sixteen hour period. Another example can be had in influenza virus. This virus has the ability to infect the embryos of chickens. Experiments show that as little as 10^{-14} grams of influenza virus introduced into a chicken embryo will initiate disease. After two days, about a milligram of virus can be recovered from such an embryo. This represents an increase from 10^{-14} to 10^{-3} grams, or an increase of about 100 billion fold. This again represents a minimum estimate of influenza virus multiplication, for in all likelihood only a fraction of the virus introduced actually plays a part in the initiation of the virus infection. This increase in the case of influenza virus required only two days. We can appreciate the magnitude of multiplication of this sort if we compare the situation with that which obtains for the human population. Evidently, the human population developed from two individuals, originally, to something like 2 billion at the present time. This represents a billion fold increase. Instead of requiring a few days, this increase required many years. The most conservative estimate is something like 6,000 years and other estimates as great as 10 million years can be found in the scientific literature. The whole point is that one cannot doubt that viruses do multiply.

This multiplication process is, in its external aspects, similar to that which one finds for living organisms. Virus multiplication might well be the result of a mechanism analogous to the cell division familiar to all biologists. In fact the 100 billion fold increase of influenza could be achieved by about 37 successive divisions into two. Since this process takes something less than two days, the rate of division would not need to be greater than once in 80

minutes. The 10 billion fold increase in tobacco necrosis virus mentioned previously, which required only sixteen hours, could be achieved by as few as 34 successive divisions of the virus particles. This would represent just slightly more than two divisions per hour. It is also possible to conceive of virus multiplication in terms of an auto-catalytic reaction. It is known for example, that pepsin can be produced from a precursor pepsinogen. This reaction is catalyzed by pepsin itself. In other words when purified pepsinogen is inoculated with a trace of the enzyme pepsin, the entire mass of pepsinogen is gradually changed over into pepsin. This is an auto-catalytic reaction. The growth of viruses in the tissues of a living host could also be a reaction of this sort. One could postulate that a single virus particle or molecule introduced into the cell of a living host can act as a specific catalyst for the synthesis of similar virus molecules present in the host. In this case, the precursors would have to be fairly simple materials - perhaps even amino acids. Which of these two alternative views of virus multiplication represents reality cannot at present be stated with any degree of assurance.

Viruses have other properties in common with living organisms. Viruses are able to undergo mutation. Even before viruses were recognized as entities, it was known that strains could exist. The practice of vaccination against small pox by infecting with a strain causing a related but milder disease dates back to the latter part of the 18th century. Pasteur, in his memorable experiments on rabies, passed rabies virus from generation to generation in the brains of rabbits. At the end of many generations, he obtained an agent which produced a disease very mild compared with the original, but which nevertheless could confer immunity to rabies. This demonstration that a virus can be transformed into a less virulent strain by laboratory manipulations also occurred before viruses were recognized as specific entities. Today it is known that many viruses can be modified by passing them through unnatural hosts. An interesting example can be had from the case of influenza virus. As was discussed previously, influenza virus has the ability to precipitate or agglutinate red blood cells of various animal species. This reaction can be made quantitative. Burnet has shown that influenza virus obtained from mouse lungs precipitates the red blood cells of mammals better than the red blood cells of birds, that is, the quantity of virus required to precipitate a fixed quantity of blood is less for mammalian blood than for avian blood. On the other hand, when this virus is cultivated in the chicken embryo for only a few generations, then it precipitates red blood cells of birds better than red blood cells of mammals. Many other instances of the modification of a virus by passage through an unnatural host could be mentioned. The production of a mild strain of dengue virus by passage through mice, recently announced by Sabin and Schlesinger, and of a non-virulent strain of yellow fever virus by passage through tissue culture as accomplished by Theiler, are examples. In both of these cases the modified virus can be used as a vaccine to immunize the individual against the more virulent disease.

It is believed by some that the modification of a virus by passage through an unnatural host is a process of selection of a strain adapted to the new host. It is believed, further, that viruses mutate frequently. When the virus is transferred to a new host, sooner or later a strain of the virus better adapted to the new host will arise by mutation. After that, this new strain will be propagated preferentially by the unnatural host. Mutations are known to occur in plant viruses as well as in animal viruses. Carsner observed that when the virus which causes the disease curley-top of sugar beets was passed through the strange plant, Chenopodium murale, a strain of the virus was selected which produced a much milder disease when taken back to the sugar beet. Johnson found that tobacco mosaic could be altered by heating. Holmes extended this observation and actually produced a strain of tobacco mosaic virus symptomless in tobacco by the simple expedient of heating plant tissues infected with the virus. McKinney was the first to study the bright yellow spots which occasionally developed on leaves of tobacco plants diseased with tobacco mosaic. An illustration of such

a leaf is shown in Figure 1. It is now known that this bright yellow spot represents a lesion produced by a mutant of tobacco mosaic virus which arose spontaneously. The development of such spots is fairly common. Jensen has isolated more than 50 strains of tobacco mosaic virus from such yellow spots. These strains are all slightly different in their biological nature, that is, they produce slightly different symptoms of various sorts. Some of these strains isolated by Jensen have been found to mutate further. At present many more than a hundred strains of tobacco mosaic virus are known.

Strong pieces of evidence for the possible molecular nature of certain plant viruses are the facts that these materials are very simple chemically and that they may be homogeneous with respect to size and shape. However, not all viruses are homogeneous chemically and uniform in size and shape. In general the viruses which cause diseases of man and animals are relatively more complex. For example, it was shown in a preceding chapter that the size of influenza virus varies about a mean. Further, this virus was shown to be made up of protein, fat and carbohydrate. There is even some reason to believe that the influenza particle has a cell membrane. In addition to that, it was shown to contain an amount of water comparable to that of ordinary living organisms such as yeast and bacteria. Somewhat similar evidence is available for vaccinia virus. It has been shown to be composed of protein, carbohydrate and fat, and in addition, to contain biotin, riboflavin, copper and other constituents of enzyme systems. This virus also contains water and behaves in some ways as though it had a cell membrane. Thus, the evidence of chemistry and physics, while supporting the hypothesis that some viruses may be molecules, also leads to the conclusion that some viruses very definitely resemble organisms more than simple protein molecules.

Whether viruses are molecules or living organisms, one of the important questions concerning them is how they multiply. Very little evidence has yet been amassed to provide an answer to this question. However, insight into the fundamental aspects of this matter can be gained from an answer to the simpler question of whether infection by a virus is induced by the interaction of the host with a single virus particle or with many virus particles. While there is disagreement concerning this matter, considerable experimental evidence can fortunately be brought to bear upon it.

Symptoms by viruses are generally of two sorts, localized and systemic. For example, in dealing with plant viruses, one can have a systemic infection such as is realized when tobacco mosaic virus is allowed to infect a Turkish tobacco plant. The entire plant shows signs of disease. Conversely, when tobacco mosaic virus is rubbed onto the leaf of a plant of Nicotiana glutinosa, a weed closely related to tobacco, necrotic lesions are formed on the rubbed leaf. The virus does not spread through the plant. In animal virus diseases a similar differentiation can be made. In a disease like influenza in the human, the symptoms and signs are general. However, in a disease such as Herpes simplex, or the common cold sore, the virus seems to produce local lesions on the face and lips.

When one inoculates a susceptible host with virus solutions at different levels of concentration, one invariably finds that at relatively high concentrations virtually all of the hosts inoculated succumb to disease. At very low virus concentration, on the other hand, virtually none of the hosts succumb, and at intermediate virus concentrations, some but not all of the host individuals succumb. When local lesions are produced by the virus, then one finds that at high virus concentrations the maximum numbers of lesions is produced; at extremely low concentrations no lesions were produced; and at intermediate concentrations intermediate numbers of lesions - depending upon the virus concentrations- are produced. This state of affairs is illustrated by some data obtained with tobacco mosaic virus. In Figure 36 can be seen four leaves of Nicotiana glutinosa.

FIGURE 36 - LOCAL LESIONS SHOWN BY N. GLUTINOSA LEAVES RUBBED WITH TOBACCO MOSAIC VIRUS SOLUTIONS OF DIFFERENT CONCENTRATIONS. (F. O. Holmes, Bot. Gaz. 87, 39 (1929)).

The leaf on the left was rubbed with a tobacco mosaic virus solution containing about a milligram of virus per cubic centimeter. The leaf next to it was rubbed with a solution 1/10 as concentrated, and so on. You can see that the number of local lesions produced is dependent upon the virus concentration. As a generalization, if one carries out a large number of experiments and then plots the number of local lesions produced or the fraction of hosts showing systemic infection against the logarithm of the concentration of virus used as innoculum, one obtains sigmoidal curves of the general shape shown in Figure 37.

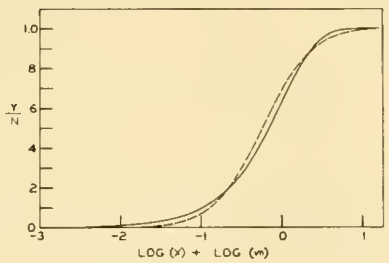


FIGURE 37 - GRAPH SHOWING PROBABILITY OF VIRUS INFECTION PLOTTED AS A FUNCTION OF LOG_{10} VIRUS CONCENTRATION. SOLID LINE DERIVED FROM LAWS OF CHANCE, BROKEN LINES DERIVED FROM HOST VARIATION THEORY. (M.A.Lauffer and W.C.Price, Arch. Biochem. 8, 449 (1945)).

There are two theoretical approaches which can lead us to curves of this shape. The first is that if one makes the assumption that a virus infection can be initiated by the chance occurrence of a virus unit in a location on the host which affords a favorable medium for that virus particle, then one can show by an elementary application of the laws of chance that the relationship between the number of infections obtained and the concentration of virus should be that given by the solid line on Figure 37. This theoretical approach is based solely upon the assumption that a single virus unit favorably situated can initiate infection. It does not in any way depend upon the number of virus units which must be applied to a host before infection is likely. For example, there is considerable evidence to indicate that infection can occur in tobacco plants only when virus comes in contact with a cell on the leaf surface which has been injured sufficiently to allow the virus particle to enter, but not sufficiently to cause the death of the cell. Thus when one is dealing with infection of tobacco plants by tobacco mosaic virus, one must consider not the total number of

virus particles which are spread upon the surface of a leaf, but rather the number of particles which come into contact with cells on the leaf surface injured sufficiently but not too much. Perhaps as few as one in a million of the virus particles spread upon the surface of the leaf actually is so favored. One might be tempted to say that it takes a million particles on the surface of a leaf to cause infection. On the contrary, only one of that million is involved in the infection process. At any rate it is possible to develop a theory of virus infection based upon the sole assumption that infection depends upon the chance occurrence of a single virus particle in a favorable region within the host. Such a theory does give a quantitative relationship between level of response and dose which agrees in a general way, at least, with common experience.

It can be shown by an application of the laws of chance that if the number of virus particles per cc in a liquid is nx, the probability that one will not find any virus particle in a small element of a volume of v cc is equal to e^{-vnx}. If the probability of not finding any particles in that volume is given by this expression, then the probability of finding at least one but maybe more than one is $1 - e^{-vnx}$. If this probability is the same as the probability of getting an infection, one can write the equation

$$\frac{y}{N} = 1 - e^{-vnx} \qquad (1)$$

where y is the number of infections observed and N is the maximum number possible. This equation was derived entirely from the laws of chance. Perhaps it can best be illustrated by considering its application to a game of chance. In the game familiarly known as craps, it is an expensive matter for the player to roll a pair of ones with the dice. Gamblers usually call this combination "snake eyes". Since a dice has six faces, there is one chance in six that a one will turn up on one dice and one chance in 36 that it will turn up in both dice. A gambler might have to roll the dice many times in sequence to try to make his point. Suppose he has to roll the dice five times in sequence. What is the probability that he will not obtain "snake-eyes" at least once? Well, if he rolls the dice one time, the probability of not getting "snake-eyes" will be $\frac{35}{36}$. If he rolls them five times, the probability will be $\frac{35}{36}$ raised to the fifth power. This latter can easily be shown to be very nearly the same as $e^{-5} \times \frac{1}{36}$. Thus the probability of not obtaining "snake-eyes" in five rolls is $e^{-5} \times \frac{1}{36}$. The probability of obtaining at least one pair of ones is

$$1 - e^{-5} \times \frac{1}{36}$$

This expression, potentially useful to crap shooters, is exactly analogous to equation (1) derived for virus solutions.

Another point of view can also lead to a similar and relationship between virus concentration and host response. This is the second alternative theory. It is argued by some that the variation in response to different levels of dosage is due primarily to variation of the susceptibility of the host to the virus. This view, when investigated mathematically, leads to a prediction of the response-dosage relationship shown by the broken curve in Figure 37. It is evident that it will require reasonably precise data to differentiate between the two theoretical possibilities. The fundamental reasoning underlying this second approach is somewhat as follows. It is postulated that the susceptibility of the host to the virus varies from individual to individual in such a way that the logarithm of susceptibility is distributed according to the normal distribution law, illustrated by the normal curve shown in Figure 38.

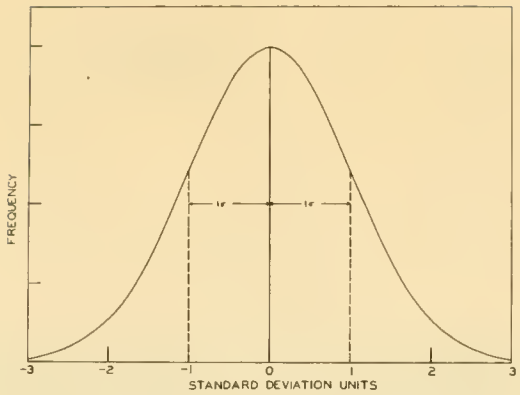


FIGURE 38 - NORMAL CURVE

If this type of distribution of susceptibility within the host actually occurs, then certainly one would expect to find that if the dosage level is low enough, virtually no host individuals will become infected. On the other hand, if the dosage level is somewhat higher then only the more susceptible of the hosts will become infected, and as the dosage level becomes greater and greater, more and more of the host individuals will become infected. Finally if the dosage level is great enough, all of the hosts will become involved, even the least susceptible. If one constructs from a distribution of this sort a theoretical relationship between the response in the host and the dosage level, one obtains the broken curve in Figure 37. The specific curve constructed was obtained for a distribution of host susceptibility in which the standard deviation of the logarithm of susceptibilities was equal to exactly 1/2 logarithmic unit. This theory of host-virus interaction has been advocated most strongly by Bryan and Beard. Its essential feature is that the host has some mechanism for combating the virus. The more strongly developed that mechanism is within an individual host, the greater the quantity of virus the individual can combat. If the dose of virus does not exceed the ability of the host to combat, then the host does not become infected. On the other hand, if the dosage is so great that the defense mechanism becomes exhausted, then the individual succumbs. Hosts vary in the extent of their defense mechanism. This theory is widely different from that outlined previously and yet it leads to an end prediction of the variation of response with dosage which differs only slightly from that of the previous theory. We will now try to examine some experimental data to determine whether or not it is possible to differentiate between these two view points.

Perhaps the best test of the theoretical deductions concerning the nature of the dose response to dose level is afforded by the study of Parker with the Board of Health strain of vaccinia virus. This strain is highly virulent for rabbits inoculated intradermally. Parker injected quarter-cc portions of virus suspension at various concentration into the rabbit skin. Severe necrotic lesions were formed. The results are presented graphically in Figure 39, where the fraction of inoculated sites showing lesions is plotted against the logarithm of virus concentration.

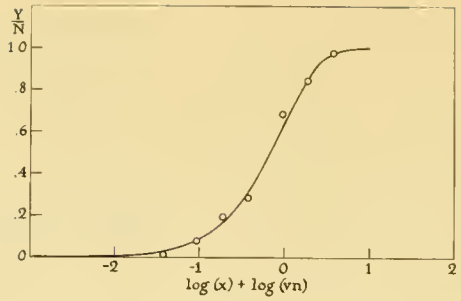


FIGURE 39 - FRACTION OF VACCINIA VIRUS INOCULATIONS THAT DEVELOPED LESIONS PLOTTED AS A FUNCTION OF LOG_{10} VIRUS CONCENTRATION. PARKER'S DATA FOR BOARD OF HEALTH STRAIN. CURVE IDENTICAL WITH SOLID LINE ON FIGURE 37. (M.A. Lauffer and W.C. Price, Arch. Biochem. 8, 449 (1945)).

The smooth curve fitting the data is the graph of equation (1), derived from probability theory on the assumption that one virus particle is sufficient to induce an infection. It can be seen that the data fit the curve beautifully. Haldane used a statistical method to evaluate the closeness of the fit of these data to the theoretical equation. His results indicate very satisfactory agreement. If one attempted to fit these data to equations derived from the assumption that a minimum of two virus particles is necessary for inducing an infection, very bad agreement would be found. These data indicate conclusively that if infection is the result of the requisite number of vaccinia virus particles being found in a favorable site, then that requisite minimum number is one particle. This is true in spite of the fact that studies carried out by Smadel, Rivers and Pickels show that on the average, about four vaccinia virus particles must be introduced into a rabbit skin in order to produce a lesion. We can interpret this by saying that it is necessary to introduce four particles because the probability is only about 25% that a particle introduced will have an opportunity to induce an infection.

Data are also available from studies on plant viruses. In the case of the plant viruses, one rubs a virus solution over the surface of a leaf. In due course, necrotic local lesions are produced on the leaf. One then counts the number of lesions present on the leaf and plots that number as a function of the logarithm of virus concentration. Data for various strains of tobacco mosaic virus on Nicotiana glutinosa leaves were obtained by Bald, by Caldwell, and by Beale. When the number of lesions expressed as the fraction of the number possible is plotted against logarithm of concentration, the data shown in Figure 40 are obtained.

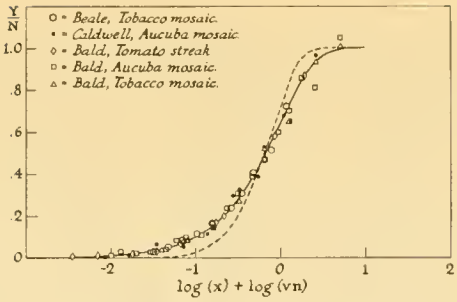


FIGURE 40 - EXPERIMENTAL PROBABILITY OF INFECTION OF N. GLUTINOSA WITH TOBACCO MOSAIC VIRUS PLOTTED AS A FUNCTION OF LOG_{10} VIRUS CONCENTRATION. SOLID LINE IS IDENTICAL WITH SOLID LINE IN FIGURE 37. (M.A.Lauffer and W.C.Price, Arch. Biochem. 8, 449 (1945)).

The solid curve fitting the data is the graph of the equation derived from probability theory for the assumption that only one virus particle is needed to cause infection. The broken curve which does not fit the data is the graph of the equation derived for the assumption that at least two virus particles are needed to cause an infection. It is clear that the data fit the equation for one particle better than that of more than one. Further data on plant viruses are available. Price studied tobacco ringspot virus on cowpeas and southern bean mosaic virus on the common bean. Chester studied cucumber mosaic virus on cowpeas, and Bald studied potato X virus on N. glutinosa. All of these data are presented in Figure 41.

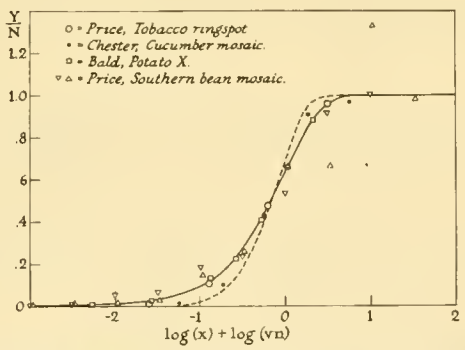


FIGURE 41 - EXPERIMENTAL PROBABILITY OF INFECTION WITH VARIOUS PLANT VIRUS-HOST SYSTEMS PLOTTED AS A FUNCTION OF LOG_{10} VIRUS CONCENTRATION. SOLID LINE IS IDENTICAL WITH SOLID LINE IN FIGURE 37. (M.A. Lauffer and W.C. Price, Arch. Biochem. 8, 449 (1945)).

In this case too the data are seen to fit the graph of the equation derived on the assumption that one virus particle can cause an infection if it happens to be favorably situated. This does not mean at all that one lesion will be produced on the surface of a leaf for every virus particle in the solution rubbed across the surface of that leaf. The rubbing spreads a film of an unknown amount of virus solution and causes numerous minute injuries. The data mean that it is necessary for only one virus particle to come into contact with a suitable injured region on the surface of the leaf.

We have seen thus far that all of the data examined show that the percentage response of a host to disease depends upon the concentration of the virus in a manner consistent with the assumption that virus infection is the result of a single virus particle coming into contact with a favorable position within the host. The data exclude absolutely the possibility that it is necessary for more than one infectious unit to come in contact with a favorable location. The fact that the data agree with the theory does not prove, however, that this mechanism is correct. It has already been seen that a curve very similar to the one which the data fit can be derived from the assumption that the differential response at various levels is due to variation of host susceptibility. Thus it is necessary to try to decide between the two theories: (1), that dosage response is due to chance occurrence of at least one virus particle in a favorable region within the host, and (2), that dosage response is due to variation of the susceptibility of hosts. There are three lines of evidence to indicate that the latter alternative is completely untenable. The first line of evidence is shown in Figure 42, which contains data obtained for 10 different viruses affecting plants and animals.

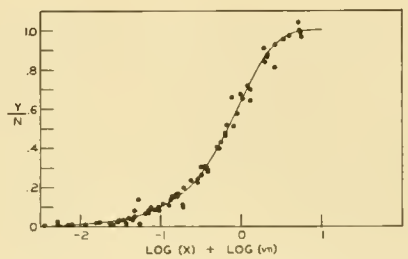


FIGURE 42 - FRACTIONAL RESPONSE PLOTTED AGAINST LOG_{10} VIRUS CONCENTRATION FOR TEN DIFFERENT VIRUSES AFFECTING PLANTS AND ANIMALS.

When the fractional response is plotted against logarithm of concentration of virus applied, all of the data can be fitted to the same curve. This is the curve of the equation derived from the assumption that one virus particle can initiate an infection if it happens to be in a favorable position. The curve which could be derived from the second alternative, that is, the theory that dose response is due to host variation, is slightly different from this curve, and the data do not fit it quite as well. This constitutes evidence that the first theory is to be preferred to the second.

The second line of evidence is also contained in Figure 42. It can be seen that 10 different viruses, each with its own host, either plant or animal, show identical concentration ranges between the region of virtually complete response and the region of virtually no response. This result is a necessity if the theory is oorrect that response is due simply to the probability of finding a virus particle in a susceptible region. However, this result is not a necessary consequence of the theory that dose response is due to variation in host susceptibility. As a matter of fact, in order to rationalize a result such as this with the host variation theory, it would be necessary to make the additional postulate that the variability in host response to viruses is the same for widely different hosts throughout both the plant and animal kingdoms. In other words, it would be necessary to assume that the variation in the susceptibility in rabbit skin to vaccinia virus is the same as the variation in the susceptibility of the tobacco leaf to tobacco mosaic virus. Such an assumption borders on absurdity. Therefore, one is justified in regarding the necessity of making such an assumption as strong evidence against the tenability of the theory that dose response is the result of host variation.

A third line of evidence can be brought to bear on the choice between the two alternative theories. If the leaf of a plant such as Nicotiana glutinosa is rubbed with a mixture of two viruses, for example, ordinary tobacco mosaic virus and the acuba mosaic strain of tobacco mosaic virus, one might expect that if infection is due to a single virus particle, the local lesions produced on this leaf ought to contain, for the most part, only one virus, either ordinary tobacco mosaic virus or the acuba strain. In fact one can calculate from the theory that virus infection is the result of at least one particle favorably situated, the proportion of lesions which should contain both viruses and the proportion which should contain either the one or the other. The reason that some lesions should contain both viruses is simple, for the theory states that a lesion is the result of one or more particles being favorably situated. One particle is sufficient, but when the virus concentration is high enough more than one particle is apt to be present. Under such circumstances infection could be the result of several particles acting simultaneously. From the simple probability theory, one obtains the result that when the dose of mixed virus applied is very concentrated, a high percentage of the local lesions should be mixtures, but when the concentration of the mixture applied is so low that only an occasional lesion is produced, the percentage of mixed infections should be extremely low - less than 1%. Similarly, it is possible to determine the percentage of mixed infections that should be expected if the host variation theory is correct. According to this theory, the host has a defense mechanism which is capable of overpowering a certain number of virus particles, but which will be itself overpowered by a greater number. Suppose, for example, that the defense mechanism of some particular host cell is capable of overpowering exactly 1000 virus units. If 1000 units of mixedivirus are applied to this particular host, no lesion will result. If 1001 units are applied, a lesion will result, and this lesion will be either tobacco mosaic virus or acuba mosaic. If 1002 units are applied, there will be two virus units left over to cause an infection, and the chances are fairly good that the resultant infection will be mixed, that is, contain both viruses. If 1010 units are applied, there will be 10 left over, and, under these circumstances, it is virtually certain that the lesion would be compound. It is evident that the concentration range between the point at which one obtains no lesions and the point at which one is almost certain to obtain mixed infection is only 1%. Thus, it is virtually certain that a lesion will be compound, if host response to dosage level is due to variation in host susceptibility. This is true even for relatively low concentrations of inoculating medium.

An actual experiment was carried out in which a mixture of acuba and tobacco mosaic viruses was spread over the leaves of Nicotiana langsdorii plants. Necrotic local lesions were obtained. These lesions were then punched out from the leaf, ground up, and inoculated onto Nicotiana sylvestris plants. This plant gives local lesions with acuba mosaic virus but systemic infections with tobacco mosaic virus. Thus, it can be used as an indicator to tell which virus is present in the lesion from the langsdorii plant. In this study it is found that when the concentration of the mixed inocula was very low, the proportion of mixed infections was very low. This result is consistent with the assumption that infection can be initiated by a single particle favorably located, but it is totally inconsistent with the assumption that dose response is an expression of variation of host susceptibility.

Thus, three lines of evidence have been brought to bear upon the question of deciding between the two theories of virus infectivity. All three indicate that the theory that dose response is an expression of variability in host susceptibility is untenable. Therefore this theory can be eliminated. The only theory that remains is that virus infection is the result of at least one virus particle being present in a favorable location. The fact that the data at present available agree with this theory does not of course prove that this mechanism is correct. It merely permits the conclusion that it is the only mechanism yet thought up which can be supported by the data at present available. On the basis of present knowledge, therefore, the proposition that one infectious unit can

cause virus infection is the most reasonable starting point for speculation concerning the nature of virus reproduction.

In the first chapter the theory that viruses are molecules was introduced, and in it and the two subsequent chapters, data were presented which were gathered as an outgrowth of this theory. In this chapter viruses have been discussed from the point of view that they are organisms. The final chapter will be predicated upon the same point of view. Many readers will readily understand my lack of reluctance to shift points of view, but a few might deplore my apparent lack of consistency. The primary function of science is the discovery, correlation and understanding of the properties of the universe. Theories are useful tools which can be employed in that task. A good theory is one which suggests new means of unlocking nature's secrets. In the field of virology, we are very fortunate in having two excellent theories, one that viruses are molecules and the other that they are organisms. Each suggests its own types of experiments to advance knowledge. Some of the fruits of the molecular theory have been reviewed in some detail. One of the most promising possibilities suggested by the organismal theory is the ultimate ability to cultivate or grow viruses on synthetic media. This would be a tremendously important achievement. Thus it is fortunate that there are two apparently conflicting hypothesis and that some people adhere to one and others to the other. It doesn't make any real difference which is right, or even if neither is right.

CHAPTER V

VIRUSES AND HUMAN WELFARE

In any discussion of the relationship of disease to human welfare, one naturally thinks first of those maladies of man which cause suffering, permanent disability and death. When we take the world view of the importance of diseases, one of the first that we think of is malaria. In spite of what is known about its control and cure, it is estimated by some authorities that even today as many as five million people die in a year of this disease. Malaria is not a virus disease. However, several virus diseases stand high on the list of killers. Though ordinarily relatively mild, influenza occasionally occurs in an extremely severe form. It is estimated that during the 1918-19 influenza pandemic, as many as 15 million people died of this malady throughout the world. Figures for the United States alone are estimated at 500,000. There was a time when smallpox was a serious and constant threat to mankind. It was not uncommon for smallpox epidemics to kill off from 10 to 90% of a population. Indeed, some of our Pilgrim fathers boasted of their own good fortune when this disease attacked and destroyed up to 90% of the Indian inhabitants of some sections of New England. During the days of the slave trade, yellow fever epidemics were common. Thousands upon thousands lost their lives to this killer in tropical cities and on board slave vessels. The mortality figures approximated 50%.

Diseases have many adverse effects upon human beings in addition to the death and destruction that they leave in their wake. It is well recognized that epidemics have often influenced the outcome of military campaigns much more than the strategy of the generals involved. Indeed, it is estimated that at the time of our own heroic defense of Bataan, 85% of our men suffered from malaria. The course of history is sometimes affected in seemingly subtle ways. There is considerable much-quoted evidence that the Tudors of England suffered from syphilis. Many people have speculated on the influence of the suspected syphilis of Henry VIII upon the course taken by English history and even upon the development of the Protestant movement in that land. The political situation in India today is influenced by the prevalence of malaria. It is believed by some that, were this one disease eradicated, India would evolve from one of the poorest nations on the earth to one of the richest, and many of the political difficulties of that country would then become adjusted, with or without the white man's guidance. Malaria and the virus disease, yellow fever, effectively prevented the French from building the Panama Canal. It was only after the Americans had learned how to conquer these diseases that they succeeded wherein the French had failed. The tremendous influence of this event upon the subsequent rise of the United States to a world power can hardly be appreciated by us today.

Many of us fail to realize that the course of human events is affected by diseases of plants and animals almost as much as by the diseases of man himself. There are two ways in which diseases of animals are related to human welfare. First, they often serve as reservoirs for human maladies. Second, the economic well-being of man, particularly his ability to supply himself with food, is often jeopardized. It might be well to recall what a terrific scourge tuberculosis of cattle has been in this country. This disease was a constant source of danger to the human population. In addition to that, it caused the death of many milk producing cattle. Tremendous sums of money were expended for its eradication. Fortunately, it seems now to be under reasonably satisfactory control. Most of us are familiar with diseases of household pets, particularly rabies and

distemper of dogs, both of them virus diseases. The former represents a constant threat to the health of human beings. A most serious virus disease of animals is the foot and mouth disease, one which causes economic losses to the extent of a million dollars or more per year in England alone.

Even though diseases of plants do not threaten mankind directly, yet they have a tremendous influence upon his economical well-being. Perhaps the greatest tragedy resulting from plant disease was the famine in Ireland in 1845-46. This famine was caused by the destruction of the potato crop by the late blight. Countless thousands of the population of Ireland died of starvation as a result of this one plant disease. The great wave of migration of Irish to this country was a direct result of this event. The present name of the common potato, Irish potato, stems from the attention called to the importance of the potato in the Irish economy by this great famine. Virus diseases of plants are also extremely important. The virus disease called curly top, which affects sugar beet plants, caused the abandonment of 10,000 acres of beet cultivation about the year 1925. Chester estimated that the virus disease, sugar cane mosaic, reduced the yield of sugar from 400,000 tons to 50,000 tons in the state of Louisiana over an 18-year period. Tobacco mosaic virus is estimated by Chester to cause a reduction of tobacco yields of from 35 to 40 million pounds annually. While this latter loss cannot be considered as affecting the nutritional well-being of man, it certainly does affect his economic status. Some of the indirect effects of tobacco mosaic virus are potentially even more serious, for this disease can be transferred to tomatoes where it causes up to a 50% loss in the crop. Certain virus diseases of the potato cause vast damage. It is estimated that the farmers of England spend between three and four million dollars annually for the importation of disease-free stock from Scotland and Ireland. Probably enough has been said to indicate the intimate relationship between the well-being of mankind and virus disease not only of man but also of plants and animals.

The remainder of this chapter consists of a discussion of several selected diseases of direct importance to mankind and of measures which have been found useful for combatting them. First let us consider diseases of plants. In general there are five important methods which might be effective in the control of plant virus diseases. The simplest and most obvious is the careful inspection of a crop and the immediate elimination of any diseased plants, thus cutting down the foci of infection. Some virus diseases of crop plants are spread through tubers or other organs of propagation. An effective control measure is to obtain carefully-inspected, disease-free seed in such cases. Many plant virus diseases are spread by insect vectors. Thus another important control measure is the destruction or at least the control of the insect population by standard agricultural processes. A fourth approach to the control of plant virus diseases is through the development of resistant, immune or carrier strains of plants. Several virus diseases of economically important plants have been brought under control by this method. A final possible means of control is one analagous to vaccination as practiced with animals. Symptomless strains of plant viruses can be selected through laboratory study. If a plant is deliberately diseases with this strain, it is immune to the natural virus. Some plants carry symptomless strains of virus naturally. For example, in this country virtually all potato plants carry a virus called the potato X virus. As far as can be told, this virus does not produce adverse effects in the potato plant. However, whenever the virus is transferred to the tobacco plant severe symptoms are detected. The process of immunizing plants by infecting them with a mild virus is relatively dangerous, because the plant carries the mild virus infection throughout its life and is, therefore, a potential source of infection for other plants which might show signs of the disease. Further, there is always danger of mutation occurring and of a virulent virus being produced. Thus, the process of vaccination of plants has never seemed very practical.

Several examples of the control of plant virus diseases by the second and fourth methods can be cited. The British have been experiencing considerable

difficulty with various virus diseases in potato. They have found that a reasonably effective method of combating these diseases is the importation of the disease-free stock from Scotland and Ireland. Such stock costs the farmers of Britain between three and four million dollars annually. When it became apparent that the disease, curley top, of sugar beets was becoming a serious threat to the sugar beet industry of this country, new varieties resistant to the curly top virus were developed. Thus the industry was saved from that one particular ravage. A similar solution was found for sugar cane mosaic. Varieties of sugar cane have been developed that are relatively resistant to this disease so that it no longer constitutes the serious menace to sugar production it once threatened to be. Had it not been for the development of virus resistant sugar beets and virus resistant cane sugar, the sugar shortage which annoyed us all during the war might have been much worse than it actually was. Efforts are now being made to control the tobacco mosaic virus by the development of resistant varieties. It will be remembered that tobacco mosaic causes a systemic infection in tobacco plants, but local necrotic lesions in Nicotiana glutinosa. From the cross - tobacco with Nicotiana glutinosa - Holmes succeeded in selecting progeny which possessed most of the ordinary properties of tobacco, but which retained the property of Nicotiana glutinosa of producing local necrotic lesions. This tobacco plant is now being crossed with commercial varieties of tobacco. Tobacco plants are available which resemble in most respects those now under cultivation, but which differ by developing local lesions rather than systemic infections when infected with tobacco mosaic. This seems to control tobacco mosaic effectively, for the chances for the spread of a virus from a single necrotic lesion are infinitely less than from a large systemically infected plant. The use of carrier varieties is practiced with some crops. A carrier variety of plant is one which will harbor a serious virus but not show adverse signs. In England there is a prevalent disease called yellow edge of strawberries. A variety of strawberry has been developed by the name of Huxley, which carries yellow edge virus but which suffers no apparent adverse effects.

The control of virus diseases of animals and man is effected by two general types of approach. Both of them are preventive or prophylactic measures. First, virus diseases may be controlled by preventing the spread of the causative agent. In those cases in which some intermediate vector, such as an insect, is responsible for the spread of the disease, the control is realized by measures directed toward the destruction of the intermediate. When the virus spreads by direct host contact, then measures are instituted to prevent such contacts. These measures may take the form of quarantine or, when diseases of animals are considered, eradication of infected hosts is often practiced. The other approach to the control of animal and human virus diseases is through the modification of the host by either active or passive immunization. Active immunization is achieved by vaccination. There are two general types of vaccination. When an active virus is introduced into a susceptible host by an unnatural route, usually a mild disease is produced but, nevertheless, antibodies to the disease are built up in the blood stream. The live virus used for such vaccinating may be the virulent strain normally present in an epidemic, but it is vastly preferable to utilize strains which are of low virulence. The other processes of vaccination consist of the introduction of a relatively large quantity of inactivated virus. Such virus is incapable of producing disease, but is capable of inducing antibody formation. Passive immunity is developed by injecting into the blood stream of a susceptible host, antibodies to the virus obtained from a host which was previously immunized. This method of combating virus diseases has been applied successfully in the case of measles. Effective therapeutic agents have not yet been found for virus diseases. No one has ever found a specific agent, such as penicillin or the sulfa drugs, which is of proved value in the treatment of a virus infection.

By utilizing measures of the sort just outlined, some of the virus diseases of domestic animals have been brought under control. Many readers are no doubt

familiar with the success which attended Pasteur's efforts to control rabies. This work was done even before viruses were recognized as distinct entities. Pasteur passed the virus from the brain of a rabid dog to the brain of a rabbit, and then passed it from generation to generation through rabbit brain. After a long series of passages, he obtained an agent which produced only a very mild disease in the dog but which, nevertheless, conferred immunity in the dog to rabies. This same virus became the basis for the treatment of human beings suspected of being infected with rabies. Distemper, another virus disease affecting dogs and other household pets, has also yielded to the laboratory approach. Distemper vaccine is now available and can be used to immunize young animals to this disease. The control of foot and mouth disease of cattle is still somewhat more crude. The English have maintained their country free of this disease by very strict quarantine regulations and by a policy of slaughtering cattle which show evidence of this disease. The British government pays out about a million dollars annually to the farmers of Britain in payment for animals condemned and slaughtered. There is very little evidence that the British public objects to this expenditure.

Probably many people are more interested in the control of human virus diseases than in the control of diseases of plants and animals. The classic story of virus disease control is that of smallpox. There was a time when smallpox was one of the most hideous common diseases of the world, but today in more civilized countries it is no longer a serious menace. This favorable change is due in a large part to the development of vaccines. The development of smallpox vaccine dates back more than a century before the recognition of viruses as separate or distinct entities. In the early part of the 18th century, a method of control was introduced into England from the Near East. It consisted of inoculating a healthy human being with the exudate from a smallpox lesion. This inoculation was done by scarifying the skin and simply applying the exudate. It resulted in development of a few pustules. However, the disease was not nearly as severe as smallpox, and it conferred an immunity toward smallpox. This control measure was dangerous because real smallpox virus was used in the inoculation and the virus could spread to healthy individuals by the natural route and cause disease. Nevertheless, it was a relatively effective procedure. It was introduced in America in 1721 by Cotton Mather. It was still a subject of bitter controversy at the time of the American Revolution. During the latter part of the 18th century, a young man in England by the name of Jenner observed that the milkmaids who came into contact with cowpox lesions on the udders of cattle seemed to be immune to smallpox. Jenner developed this observation and introduced the practice of vaccinating human beings against smallpox with material derived from the cowpox disease. The idea was received with considerable hostility in both England and America. Nevertheless, it did gain gradual acceptance. The American Indians were among the early ones to adopt Jenner's vaccination, and they demonstrated their gratitude to the developer of the process by sending him gifts. Napoleon vaccinated his entire army against smallpox. Perhaps one of the few accomplishments really to the credit of Napoleon was his utilization of this advancement in science. Today vaccination against smallpox is almost universally practiced in civilized countries. The vaccines now available may or may not have been derived from the cowpox originally used by Jenner. There is some possibility that the present vaccinia virus, which is the material used for smallpox vaccines, may have been derived from a mutation of smallpox virus selected and developed by the cow.

The conquest of smallpox did not have to await the advancement of knowledge concerning the nature of viruses. However, most other virus diseases that have been brought under control have been studied first in the laboratory. Yellow fever affords a striking example. Many are familiar with the heroic story of the field experiments of yellow fever carried out by the Army Yellow Fever Commission. The name of Walter Reed is associated in many minds with these experiments. Through them it was demonstrated that yellow fever is transmitted from

diseased patients to healthy patients by means of the mosquito vector, Aedes aegypti. The earliest method of control of yellow fever involved simply the destruction of the mosquito. Since this insect is one which breeds near the habitation of man, it proved to be not too difficult a problem to eradicate it in populous areas where epidemic yellow fever hitherto had been prevalent. Thus yellow fever as an epidemic was conquered. However, yellow fever was not eradicated by this approach. Gradually the realization grew upon medical scholars that yellow fever still persists in the jungles and that occasionally men exposed to the jungle habitat become infected. Thus it became necessary to find some other means of combating the danger of yellow fever for those individuals who are required to spend time in the jungles of tropical regions. Clearly, insect control was not the answer, for it is impossible to eradicate all the possible insect vectors in a jungle. Progress toward the solution of the yellow fever problem was realized when it was first found possible to develop the disease in experimental animals. The disease was first transferred to the rhesus monkey in 1927 by Stokes, Bauer and Hudson, and later - in 1930 - it was shown by Theiler that it could be passed to the mouse by intracerebral inoculation. Later the virus was propagated in tissue culture and finally by Elmendorf and Smith in chicken embryos. Numerous attempts were made to attenuate the virus by successive passages through mouse brain and other materials. Finally Theiler and associates developed a strain of yellow fever virus now known as 17D. This strain was obtained by serial passage in chicken embryo tissue culture from which the central nervous system was removed. It turned out to have the remarkable property of producing an extremely mild infection in man which nevertheless confers complete immunity against yellow fever. Thus today a live vaccine is available for use against yellow fever. Millions of people have been vaccinated by 17D. The success of our armed forces in jungle regions must be attributed in large part to the immunity to jungle yellow fever derived from this one vaccine. Attempts have been made by Theiler to reproduce the development of a strain of yellow fever similar to 17D. Thus far they have been without success. This development can be considered as a laboratory accident. Nevertheless it has provided a completely acceptable means of protecting against yellow fever for those in the jungles.

Other virus diseases have yielded to a similar experimental approach. One of the most recent is dengue. This is a serious tropical regions disease which causes a severe rash in the individual and a total disability for the duration of the attack. During the war, the Dengue Commission of the U.S. Army gathered dengue virus from many theatres of the war both in the Mediterranean and the Pacific areas. Large scale experiments were carried out with human volunteers in this country by Sabin and Schlesinger, at that time of the Army Medical Corps. Human volunteers were used for perpetuation of the virus and for test subjects to determine the success or failure of attempts to grow the virus in experimental animals. Eventually Sabin and Schlesinger were able to get the virus to infect a particular strain of white mice. They achieved success only after they first used the physical method of ultracentrifugation to concentrate the virus and thus provide a high concentration level. This was passed through many generations of mice, and eventually a strain was isolated which has the remarkable property of producing only the skin rash characteristic of dengue. The fever and general disability associated with the virus are not produced. Antibodies to the virulent form are induced. This live virus can be used as a vaccine to protect troops who must enter areas where dengue is prevalent. Its development virtually coincided with the end of the war. Thus far, no satisfactory test of its effectiveness has been realized. However, there is every reason to believe that dengue vaccine will prove an effective prophylactic agent against the ravages of dengue.

Influenza is another of the virus diseases to have gone down in defeat before the forces of science during the late war experience. Influenza has been a serious source of death at various times during the past. It is not probable that the high death toll experienced during the pandemic of 1918-19 will ever

again threaten, because death usually is the result of complications such as pneumonia following the attack of influenza. The methods available for combating pneumonia probably are adequate to insure against a repetition of the terrific death toll. Nevertheless influenza is a serious disease which, even when it does not kill, completely disables its victims for a period of one to four weeks. It takes little imagination to picture the consequences of an influenza epidemic in an active military theater. It was an urgent war problem to develop a satisfactory influenza vaccine. It will be remembered that the second way of vaccinating is to introduce into the body of the host a large amount of suspension of virus rendered incapable of multiplying and producing disease, but not incapable of inducing the formation in the blood of antibodies. Vaccination against influenza is of this type. As a result of fundamental biological studies in influenza virus carried out in several laboratories, and of chemical and physical studies carried out at the Rockefeller Institute for Medical Research, two types of influenza vaccine were developed during the war. Both have been used to immunize the members of our armed forces and both are now available for civilian use. Both of these vaccines are composed of suspensions of influenza virus to which an amount of formaldehyde has been added, sufficient to destroy the ability to induce antibody formation.

The virus subsequently to be used as vaccine is grown in chicken embryos. Ten-day old chicken embryos or half-hatched eggs are inoculated with influenza virus. This is done by simply punching a hole in the shell above the air sack and introducing a hypodermic needle into the fluids surrounding the embryo. During the course of 48 hours the virus multiplies and causes a disease in the embryo. The virus is then liberated into the fluids. When the embryo is chilled, these fluids can be removed. The virus is then recovered and is treated with formaldehyde to form the vaccine. The two vaccines at present available differ with respect to the method used for the concentration of the virus. The vaccine which was first accepted by the Army is purified and concentrated by precipitation with chicken red blood cells. The precipitated red blood cell-virus complex is suspended in a small volume of salt solution and then heated up to body temperature. At this temperature the virus dissociates from the cells. The red cells can be removed by low speed centrifugation. Formaldehyde is then added to the virus to destroy its ability to reproduce. This material is distributed as vaccine. The second type of vaccine was developed as a result of the fundamental physical and chemical studies on the nature of influenza virus carried out at the Rockefeller Institute for Medical Research. In this particular case, the fluids obtained from the diseased chicken embryos are subjected to high speed centrifugation with a Sharples Supercentrifuge - a device very much resembling an ordinary cream separator. The formaldehyde used to inactivate the virus is added to the fluid before centrifugation. The inactivated virus collects on the periphery of the centrifuge in a mass. This can be suspended in a small volume of salt solution. The centrifugation process seems to give a somewhat higher degree of concentration of the virus and produces a product which causes a low incidence of unfavorable reactions in the vaccinated person. There are two kinds of influenza recognized today, influenza A and influenza B. Immunity to influenza A does not protect against influenza B and conversely. Therefore influenza vaccines are mixtures of both influenza A and influenza B viruses.

The vaccine solution is administered by subcutaneous injection. When the centrifuged vaccine is used, about 3/10 of a milligram of virus is used as a dose. This represents about half of the material that can be obtained from the fluids of a single infected chicken embryo. An individual thus vaccinated produces antibodies to influenza. This can be demonstrated in the laboratory, for the serum from a vaccinated individual will neutralize virus in a test tube. This antibody formation is associated with protection against the virus disease. Henle, Henle and Stokes showed that human volunteers who had been vaccinated in this manner showed a much lower susceptibility to experimental influenza than nonvaccinated individuals. The Influenza Commission of the U. S. Army conducted a large scale test of the effectiveness of the influenza vaccine. During the

winter of 1943-44, a study was carried out with about 12,000 members of the armed forces assigned to ASTP units in colleges and universities located in many sections of the country. About 6,000 of these were inoculated with influenza vaccine and about 6,000 with salt solution. Within a few weeks after the completion of the vaccination an influenza epidemic spread across the country. When the incidence of influenza was studied in both the vaccinated group and the control group inoculated with salt solution, it was found that 7.11% of the 6,000 unvaccinated individuals developed clinically recognizable influenza. On the other hand, only 2.22% of the vaccinated group developed influenza. Thus, this experiment demonstrated that the odds against developing influenza were increased by a factor of more than 3 to 1 by this vaccine. Studies carried out by Hirst, Rickard and Friedewald show that even a year after vaccination the incidence of influenza is 1/3 less than in unvaccinated controls. These favorable statistics leave little doubt that the time is near at hand when influenza can be regarded as one of the conquered diseases.

Another disease which can be listed among the vanquished is measles. The approach to the conquest of measles is entirely different from that utilized for any other virus disease considered in this chapter. Measles is a very widely-spread disease. The vast majority of the people have had it at one time or another. Ordinarily it is not particularly dangerous, but a certain fraction of individuals develop complications which can be of a very serious nature. Thus, the control of measles is a matter of considerable importance. The fact that measles is of almost universal occurrence provides the means for controlling the disease. Virtually all normal adults are immune, that is, they have within their blood stream antibodies to measles. Experiments carried out during several years have shown that the course of a measle infection can be modified very considerably by the injection of serum from patients recently recovered from the disease. Such serum is relatively high in measle antibody content.

Studies carried out in many laboratories over the past 20 years have gradually shed light upon the composition of human blood. It has been found that the fluid component is made up essentially of five proteins. These are called albumin, fibrinogen, alpha-globulin, beta-globulin and gamma-globulin. The electrophoresis apparatus was one of the most effective tools for resolving the constituents of blood. It was learned in the course of these studies that the antibodies to disease are generally associated with the gamma-globulin fraction. During the war, when human blood was collected in hugh quantities by the Red Cross primarily for the treatment of shock, an attempt was made at the Harvard laboratories and elsewhere to devise means for the isolation from this human blood of the constituent effective for shock. This constituent is serum albumin. These fractionating procedures provided means for separating the albumin from the other constituents which play only a minor role in the control of shock. Therefore, these constituents were made available for other purposes. Since almost all human beings are immume to measles, it was only natural to determine whether or not those fractions of the processed human blood richest in gamma-globulin would be an effective means of controlling measles. This turned out to be the case. Gamma-globulin has been made available to the civilian public. Several fairly extensive studies have been carried out. In one study conducted in Boston, it was shown that the gamma-globulin fraction could be used to prevent entirely the development of measles in exposed children or it could be used to modify the course of the disease in order to produce only a mild infection. It was found that if 1/10 cubic centimeter of the gamma-globulin rich fraction of processed blood was injected per pound of body weight, the individuals will be completely protected. A quarter of this amount will result in a very mild disease. In another study carried out in New York City on 814 exposed individuals, 2 cc. of gamma-globulin was administered uniformly to patients between six months and six years of age. Sixty-five individuals in a control group received no gamma-globulin. The results are listed in Table VI. They demonstrate conclusively the effectiveness of gamma-globulin for the prevention or the modification of measles.

TABLE VI

THE DEVELOPMENT OF MEASLES IN EXPOSED SUBJECTS

Response	814 Patients Treated with gamma-globulin	65 Patients Not Treated
Regular Measles	0%	48%
Moderate Measles	2%	18%
Mild Measles	19%	17%
Complete Absence	79%	17%

Many medical authorities feel that it is desirable to use only small amounts of this gamma-globulin in patients exposed to measles. It is reasoned that the small amounts will allow the individual to develop measles, but will protect him against any serious manifestation and consequences. An immunity will therefore be generated which, it is hoped, will prove as lasting as that acquired in a normal infection.

Several virus diseases which have been overcome by the application of the scientific method have been discussed. Anyone who has any connection with research in the medical sciences rejoices in the conquest of any disease. It should be emphasized however that in every case discussed, excepting the case of smallpox, the conquest of the disease was the by-product of fundamental academic research directed primarily toward the characterization of virus as an entity. The case of influenza can serve as an example. The developing of an influenza vaccine was the by-product of physical, chemical and biological studies aimed at the characterization of this virus. These studies were made possible by the fundamental investigation on tobacco mosaic virus which followed the crystallization of that virus by Stanley in 1935. This crystallization could be achieve, however, only as a result of the experience amassed in the crystallization of other biological proteins such as enzymes. Yet who could ever have dreamed that the crystallization of urease and of pepsin would open the way for the conquest of influenza? The crystallization of enzymes can be classified as useless academic research. That being the case, it seems fitting to conclude the Priestly Lecture Series for 1946 with the expression of the hope that I have been able to communicate some of my enthusiasm for useless academic research.